JOURNAL OF AGRICULTURAL RESEARCH

VOL. XXXI WASHINGTON, D. C., NOVEMBER 1, 1925 No. 9

STUDIES ON OPHIOBOLUS GRAMINIS SACC. AND THE TAKE-ALL DISEASE OF WHEAT [1]

By RAY J. DAVIS [2]

Formerly Field Assistant, Office of Cereal Investigations, Bureau of Plant Industry, United States Department of Agriculture

INTRODUCTION

This paper deals with laboratory, greenhouse, and limited outdoor studies on the take-all disease of wheat and the causal fungus. Since this disease does not occur in the region where these studies were made, it was not practicable to make extensive field studies.

MATERIALS AND TECHNIQUE

ORGANISM

Three single-spore strains of *Ophiobolus graminis* Sacc., isolated from diseased plants growing in widely different regions, were supplied the writer by H. H. McKinney, of the United States Department of Agriculture, and these have been used throughout the investigations. One of these strains came originally from R. S. Kirby, of Cornell University, who isolated it from a wheat plant affected by take-all in New York State. This strain is referred to throughout this paper as the "New York strain." The other two strains were isolated by Hurley Fellows, of the United States Department of Agriculture, from take-all diseased wheat plants growing near Hillsboro, Oreg., and Fayetteville, Ark., respectively, and are referred to throughout the paper as the "Oregon strain" and the "Arkansas strain."

MEDIA

Preliminary experimentation dealing with the growth and sporulation of the organism on different media led to the adoption of the following three media for the greater part of the work: (1) potato-dextrose agar, (2) string-bean agar, and (3) cooked kernels of barley and oats (equal portions).

In the physiological experiments potato-dextrose agar was used as the principal substratum. Nutrient broth and Czapek's full nutrient solution also were employed in the studies on the growth of the fungus in relation to hydrogen-ion concentration.

Potato-dextrose agar was prepared, according to the usual laboratory method, as follows: Two hundred grams of peeled potatoes were cut into small pieces, placed in 1,000 c. c. distilled water, and steamed

[1] Received for publication Feb. 2, 1925; issued January, 1925. These studies were carried on cooperatively by the Office of Cereal Investigations, U. S. Department of Agriculture, and the Wisconsin Agricultural Experiment Station, Madison, Wis.

[2] The writer wishes to express his appreciation to L. R. Jones, James G. Dickson, and H. H. McKinney for their many valuable suggestions during the progress of these studies, and to Mr. McKinney and A. G. Johnson for assistance in revising the manuscript.

in an Arnold sterilizer for approximately an hour. The liquid was then strained off and the original volume of liquid restored by addition of distilled water. Agar 20 gms. and dextrose 20 gms. were then added and steamed a short time, and filtered while hot through absorbent cotton. Equal quantities of the medium were then placed in flasks and sterilized in an autoclave for 30 minutes at 10 pounds pressure. This medium was chosen as a standard and all stock cultures were grown on it.

The nutrient broth was made by adding three grams of Liebig's beef extract and 10 gms. of peptone to 1,000 c. c. of distilled water; the mixture was warmed and stirred until the peptone was dissolved, then filtered, placed in flasks, and sterilized in an autoclave for 30 minutes at 10 pounds pressure.

Czapek's full nutrient solution was made according to the following formula:

Magnesium sulphate ($MgSO_4$)	gm.	0.5
Potassium dihydrogen phosphate (KH_2PO_4)	gm.	1.0
Potassium chloride (KCl)	gm.	.5
Sodium nitrate ($NaNO_3$)	gm.	2.0
Ferrous sulphate ($FeSO_4$)	gm.	.01
Sucrose ($C_{12}H_{22}O_{11}$)	gm.	30.0
Distilled water to make	c. c.	1,000

Equal quantities were placed in flasks and sterilized for 30 minutes at 10 pounds pressure. The methods employed in adjusting the various media to different hydrogen-ion concentrations are described later under the heading of the influence of hydrogen-ion concentration on growth of the fungus.

String-bean agar, which proved to be most satisfactory for perithecial production, was prepared in the same manner as potato-dextrose agar, except that string beans were substituted for potatoes and the dextrose was omitted.

A medium made from equal parts of oats and barley kernels proved to be favorable for increasing the fungus for purposes of inoculating soil. This medium enabled the fungus to make a luxuriant growth, and very little if any deleterious effect on the wheat plants resulted when the uninoculated medium was used in the soil in the controls. The grain mixture for this medium was first soaked over night in cold water and then boiled in an excess of water over a free flame for about an hour. Then 500 and 1,000 c. c. of the cooked kernels were placed in Erlenmeyer flasks of 1 to 2 liters capacity, respectively. Enough of the decoction water was added to each flask to keep the kernels moist, but not too wet, throughout the period the fungus was growing. The flasks were plugged with cotton and sterilized 45 minutes at 15 pounds pressure.

Following the inoculation, and after the fungus had thoroughly grown through the mass of kernels, the contents of the flasks were used to inoculate experimental soil. An equal quantity of inoculum was added to and throughly mixed with the upper soil in each container. Likewise in controls an equal quantity of the uninoculated medium was added. Immediately following this treatment wheat was sown in the inoculated and uninoculated soil.

Other media, especially cooked wheat kernels, produced a somewhat better growth of mycelium, but it was found that these media were toxic to wheat plants.

A modified Crone's nutrient agar, made according to the following formula, was used as the medium in which to grow wheat plants under aseptic conditions:

Potassium nitrate (KNO_3)	gm.	1. 0
Magnesium sulphate ($MgSO_4$)	gm.	0. 25
Calcium sulphate ($CaSO_4$)	gm.	. 25
Calcium phosphate ($Ca_3(PO_4)_2$)	gm.	. 25
Iron sulphate ($FeSO_4$)	gm.	. 25
Distilled water to make	c. c.	1, 000. 00
Agar	gms.	10. 00

SEEDS

Three varieties of wheat were employed in these experiments, namely, Goldcoin (Junior No. 6), California Club, and Marquis. The first named was used in the greater part of the work. The kernels were, in all cases, sterilized 10 minutes in 1–1000 $HgCl_2$, washed thoroughly in running water, dried at room temperature, and planted 1½ inches deep at the rate of 1 gram per linear foot.

SOIL

The soil used in all of the experiments was a silt loam, obtained at the base of a hill covered with trees and shrubs, and was, therefore, reasonably free from general field-crop soil-infesting organisms. It was black, very rich in organic matter, and had a hydrogen-ion concentration of about 6.6 P_H after sterilization for 2 hours at 15 pounds pressure.

TAXONOMY OF THE FUNGUS

The taxonomy of the take-all fungus is discussed by McKinney (*10*)[3] and is not taken up in detail here. The parasite was described as *Ophiobolus graminis* by Saccardo (*14, p. 349*) and this name has been generally accepted. Recently Fitzpatrick, Thomas, and Kirby (*5*) concluded that this name is invalid. However, as pointed out by McKinney (*10*), there seems to be justification for continuing to use Saccardo's name and therefore the writer is following this older and generally accepted usage.

MORPHOLOGY OF THE FUNGUS

Throughout the course of these studies numerous mature perithecia were produced by the New York strain of the fungus when grown in pure culture. Perithecia were also produced by this strain on wheat plants grown under controlled experimental conditions. These fruiting bodies varied greatly in size and shape, but the most of them were typical for *Ophiobolus graminis*. They were always black in color, had loose strands of mycelium about them, and averaged about 300 microns in diameter; most of them had beaks, which were usually curved. However, the beaks were extremely variable in length and shape.

Asci were produced in abundance in pure culture. They came out of the perithecium singly, and as they left the ostiole they were shot out for a short distance. The ascus wall apparently dissolved, usually beginning at one end, when it came in contact with the water,

[3] Reference is made by number (italic) to "Literature cited," p. 825.

and in less than an hour the spores began to separate. No other method of spore liberation was noted.

Spore germination was studied, but only one method was found to occur. In from six hours to two or three days, germ tubes developed and branched and rebranched, and soon formed a network of hyphae. The end cells of a spore germinated most frequently, but any cell

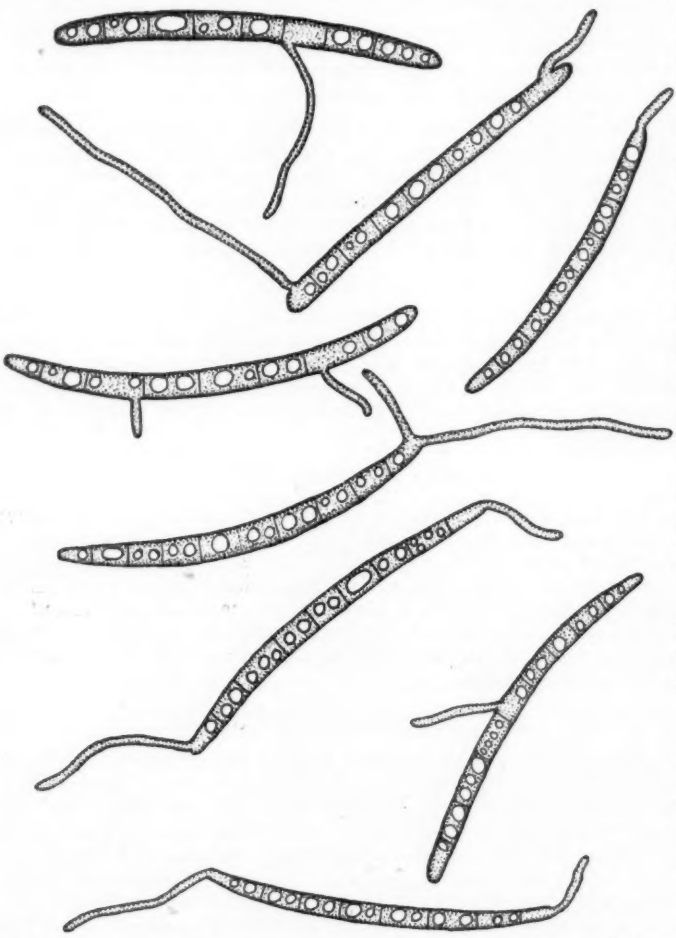
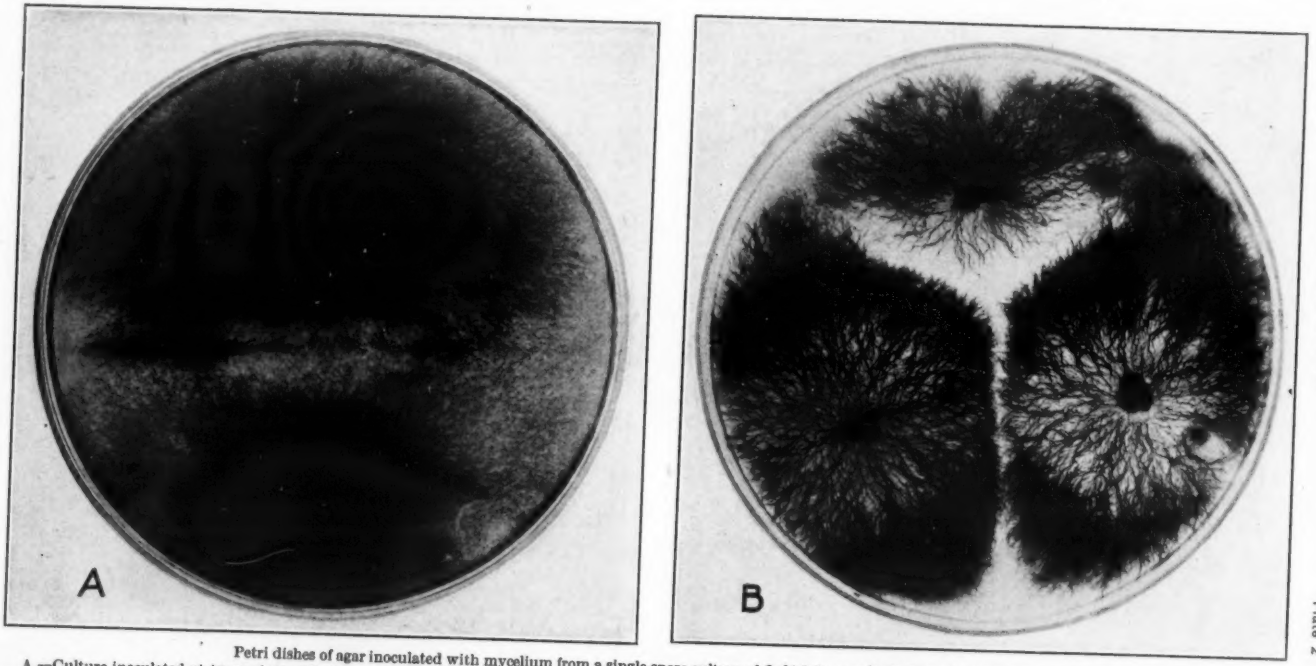


FIG. 1.—Germinating ascospores of *Ophiobolus graminis*, showing germ tubes coming from various cells of the spore. Drawn with aid of camera lucida. × 1,000

of the five to nine present was capable of germination (fig. 1), and as many as three were seen to germinate in a single spore. Hori (*6*) claims that only the end cells of the ascospore germinate, but the writer repeatedly noted that the other cells produced germ tubes.

Mangin (*12*) claims that on germination the spores form a promycelium which bears sickle-shaped sporidia, and that these give rise to germ tubes which enter the host plants through the root hairs.

A.—A single spore culture of the New York strain of *Ophiobolus graminis*. Mature perithecia can be seen in concentric circles about the point of inoculation
B.—A Petri-dish culture of *Ophiobolus graminis*, inoculated at three points, with as many different strains, as follows: (1) New York, single-spore, strain; (2) Oregon strain; (3) Arkansas strain. [Note the perithecia in the colony of the New York strain, which examinations revealed to be mature]



Petri dishes of agar inoculated with mycelium from a single-spore culture of *Ophiobolus graminis* (New York strain)

A.—Culture inoculated at two points with the same single-spore strain, and incubated in diffused light. Mature perithecia were produced at or near the line of contact of the two colonies

B.—Same as A, except three inoculations were made and the culture grown in darkness. (Note the absence of perithecia and the characteristic type of hyphae massed in compact strands. These strands are similar to those which occur in the mycelial plates under the leaf sheaths of infected wheat plants)

However, Delacroix (*3*) made further observation in an attempt to verify Mangin's work, but failed to find promycelia and sporidia. He suggested that Mangin probably had another species of Ophiobolus.

In the course of the present studies, no spore forms other than ascospores have been found. Paraphyses were present in the perithecia during ascus formation, but as the spores matured they disappeared. Perithecia in which the asci were well developed, but in which no spores were differentiated, contained paraphyses. The paraphyses were hyaline, three to four celled, slightly broader at the base than at the tip, and rather irregular in shape. When the ascospores were formed, the paraphyses disappeared. Apparently they were absorbed to furnish food for the developing spores, as no trace of them could be seen in any fully matured perithecium.

The mycelium was found to be extremely variable both in general appearance and diameter of hyphae, but this was probably due in part to age and conditions of growth. In rapidly growing cultures a fine, hyaline mycelium with granular protoplasm and few or no cross-walls was very abundant. As the hyphae became older, they increased in diameter, the walls became brown in color, and numerous

FIG. 2.—Fine hyaline mycelium developed at a broken end of a coarse brown hypha of *Ophiobolus graminis*. Drawn with aid of a camera lucida. × 1,000

septa were formed, the protoplasm lost its granular appearance, and oil droplets appeared in the cells. Furthermore, an injury to a coarse hypha often would induce the formation of a new hypha at the point of injury. This new mycelium invariably had a much smaller diameter than the old, as shown in Figure 2. With ordinary staining methods the fine mycelium readily took up and retained the stain, while the coarse type did not hold the stain well. Very often the mature hyphae adhered closely together and formed strands. As many as 6 hyphae have been seen united in this manner, and when attempts were made to separate them the whole strand often broke crosswise.

As stated previously, pure cultures of the New York strain of the fungus formed numerous mature perithecia when grown on artificial media. However, the Oregon and Arkansas strains of the parasite never produced mature fruiting bodies when handled in the same manner as the New York strain. In some cases perithecium-like bodies were produced, but these were never found to contain asci or ascospores. When the Oregon and Arkansas strains were planted on opposite sides of a Petri dish containing solid medium, they seldom grew together. When they did, however, the hyphae were much darker in color along the line where the two mycelia met than elsewhere, but in no case did mature fruiting bodies develop in these dishes. Likewise each strain was planted in Petri dishes with the New York strain, and also all three strains were planted in the same dish. Dark lines developed at the juncture of the colonies in each case, but perithecia were produced only where the New York strain touched the other strains and in concentric circles about the center of inoculation in the colonies of this strain (pl. 1, B).

Numerous solid media and decoctions were used in the attempt to obtain sporulation in the Oregon strain, but none of these gave positive results. Hydrogen-ion concentration, temperature, light, moisture, and aeration also were varied, but with negative results. Possibly these cultures are sterile, but the writer is inclined to think that if the right conditions were found they would sporulate. Both the Oregon strain and the Arkansas strain were isolated from ascospores. This, together with the fact that they formed immature fruiting bodies at several times, would indicate that they would form mature perithecia under the proper conditions.

The New York strain fruited intermittently, but only after the writer had worked with it for several months. When perithecia formed they were abundant, but at times the fungus would go for several months without fruiting. Then suddenly many of the fresh transfers would form perithecia. The uncertainty of perithecial formation was still more evident when a large number of plates and tubes were inoculated at one time. Although the greatest care was exercised to obtain uniform conditions in any one series, there would invariably be a few cultures which would not develop perithecia. Evidently there is some important factor influencing sporulation that has not yet been determined.

It is reported by Kirby (*8*) that all of the New York strains of *Ophiobolus graminis* isolated exhibit the phenomenon of heterothallism. He states that in order to get sporulation it is necessary to grow plus and minus strains together. However, under the conditions of the present work the New York strain, which came originally from Kirby, was always homothallic. Single ascospores were frequently isolated, and these were germinated and grown separately. These single-spore cultures produced mature perithecia repeatedly in Petri-dish cultures without the influence of mycelium from any other strains.

Further, when portions of mycelium from a monosporous culture were placed on opposite sides of a Petri dish containing string-bean agar, a line of dark-colored mycelium was formed where the two mycelia came together, and perithecia were developed along this line (pl. 2, A). In this case, two colonies from the same ascospore acted in the same manner as mycelia from the so-called plus and minus strains reported by Kirby (*8*). This experiment indicates that two advancing masses of hyphae may change the nature of the substrate to the extent that sporulation may be aided, and in this case induced along the line of contact of the colonies. In an environment that is not favorable for sporulation, or when weak cultures of the fungus are used, two strains may induce sporulation where otherwise they would not fruit. It is evident from these results that nutrition or the products of metabolism may act as a stimulant for sporulation. Possibly this is the explanation of the results obtained by Kirby. It is the opinion of the writer that the lack of sporulation of the Oregon and Arkansas strains of the fungus in the present studies is probably caused by their extreme lack of vigor. Due to their long culture on artificial media, with mycelium used for transfers, their reproductive ability probably became so low that the various stimuli used could not restore it. It seems entirely possible that when other factors which influence sporulation are determined these cultures might be made to sporulate.

INFLUENCE OF TEMPERATURE ON GROWTH OF THE FUNGUS

The parasite was grown at different temperatures on 2 per cent potato-dextrose agar in Petri dishes, and the growth was determined by measuring the diameter of the colonies. The agar used had a hydrogen-ion concentration of 7 P_H, and in all cases the same amount of medium was used in equal-sized Petri dishes. Young, vigorous stock cultures growing in Petri dishes were employed for supplying the transfer material. In order to insure uniformity among all the transfers, blocks of agar 1 mm. square containing mycelium were cut at equal distances from the center of the stock colony and placed in the center of each Petri dish filled with fresh agar. The dishes

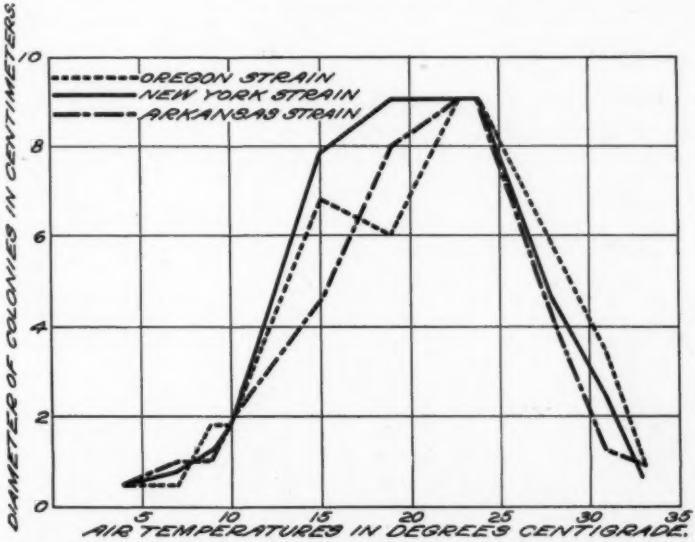


FIG. 3.—Influence of air temperature on the growth of mycelium of three strains of *Ophiobolus graminis*. Data from Table I

were then placed in the various temperature compartments of an Altmann incubator. The chambers of the incubator were kept as nearly as possible under the same conditions, except temperature. These chambers excluded all light when closed, hence the growth was in darkness. An exception to this was necessary in the case of the highest temperature in the first four experiments and in the case of the 28° C. temperature in the fifth experiment. These high temperatures were maintained in a separate incubator which contained a dim electric light. As this light did not strike the plates, directly, and as it was determined, as reported later in this paper, that light of that intensity had very little inhibitory influence on mycelial growth, it seems evident that this weak illumination did not impair the value of the temperature results obtained in this case.

Ten series with the Oregon strain, and four series each with the New York and Arkansas strains, were studied. In each experiment

there was a gradual increase in growth from the minimum temperature to the optimum, then a more rapid decrease as the maximum temperature was approached. A temperature of 36° C. was available in only the first four experiments, but growth of all cultures kept at this temperature in these experiments was entirely checked. The lowest temperature employed was 4° C., at which growth was exceedingly slight. Measurements of diameter of colonies of the different strains grown at the different temperatures are given in Table I and the averages are shown graphically in Figure 3.

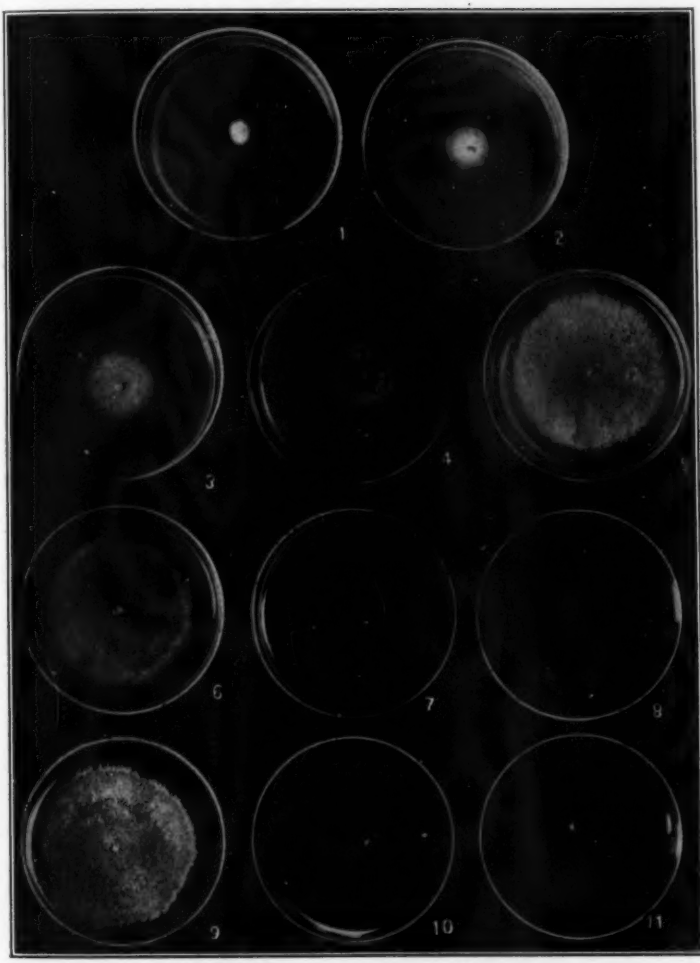
TABLE I.—*Effects of air temperatures on the growth of the mycelium of three strains of Ophiobolus graminis on potato-dextrose agar, as shown by measurements of diameter of colonies*

Strain of fungus	Diameter of colonies grown at the different temperatures										
	4° C.	7° C.	9° C.	10° C.	15° C.	19° C.	23° C.	24° C.	28° C.	31° C.	33° C.
	Cm.	*Cm.*	*Cm.*	*Cm.*	*Cm.*	*Cm.*	*Cm.*	*Cm.*	*Cm.*	*Cm.*	*Cm.*
Oregon	0.5	0.5	2.0	2.0	6.5	6.0	9.0	9.0	------	2.5	1.0
	.5	.5	2.0	2.0	7.0	6.0	9.0	9.0	------	2.5	------
	.5	.5	1.5	1.5	7.0	6.0	9.0	9.0	------	4.0	------
					6.5	6.0	9.0	9.0	------	4.5	------
Average	.5	.5	1.8	1.8	6.8	6.0	9.0	9.0	------	3.4	1.0
New York	.5	1.0	1.5	2.0	8.0	9.0	9.0	9.0	6.5	2.5	.5
	.5	1.0	1.5	2.0	8.0	9.0	9.0	9.0	4.0	2.5	.5
	.5	.5	1.0	1.5	8.0	9.0	9.0	9.0	3.5	2.5	1.0
					7.0	9.0	9.0	9.0	------	2.0	------
Average	.5	.8	1.3	1.8	7.8	9.0	9.0	9.0	4.7	2.4	.7
Arkansas	.5	1.0	1.0	2.0	5.0	8.0	9.0	9.0	4.0	2.0	1.0
	.5	1.0	1.0	2.0	5.0	8.0	9.0	9.0	4.5	2.0	1.0
	.5	1.0	1.0	2.0	4.0	8.0	9.0	9.0	------	.5	1.0
					4.0	8.0	9.0	9.0	------	.5	.5
Average	.5	1.0	1.0	2.0	4.5	8.0	9.0	9.0	4.3	1.3	.9

A typical complete series of cultures is shown in Plate 3. It is evident that *Ophiobolus graminis* will grow at temperatures ranging from 4° to 33° C., with a maximum growth at 23° to 24°. This range of temperature for the growth of the fungus is almost as wide as the range for growth of the wheat plant, and even though wheat will grow at a slightly higher temperature, it can not be grown economically under such conditions. The optimum temperatures for the growth of both are about the same, as shown by McKinney and the writer (*11*) but it can not be assumed from this that the fungus is most destructive to wheat plants at those temperatures.

INFLUENCE OF HYDROGEN-ION CONCENTRATION ON GROWTH OF THE FUNGUS

Growth for the three strains of the organism as related to hydrogen-ion concentration was studied in potato-dextrose agar, nutrient broth, and Czapek's full nutrient solution. In the solid medium, growth rates were determined by measuring the diameter of the colonies. In the liquid media, the dry-weight method was used. The hydrogen-ion concentrations of the media were determined by the Clark and Lubs (*2*) colorimetric method. The determinations were made at room temperature, following adjustment of the media to a desired reaction, and such values represent the initial hydrogen-



Petri-dish cultures of *Ophiobolus graminis* on 2 per cent potato-glucose agar, incubated at different temperatures as follows: 1, 4° C.; 2, 7°; 3, 9°; 4, 10°; 5, 15°; 6, 19°; 7, 23°; 8, 24°; 9, 28°; 10, 31°; 11, 33°

A.—Goldcoin wheat plants 30 days old, showing a severe case of take all. These plants were grown under greenhouse conditions in soil inoculated with a pure culture of the Oregon strain of *Ophiobolus graminis*

B.—Healthy plants of same age and same variety, grown in uninoculated soil

ion concentration. In the solid medium, only initial determinations were made, but in the liquid media both initial and final PH values were determined. As soon as possible after adjusting the medial they were inoculated with a uniform quality and amount of inoculum, namely, young, vigorous mycelium on blocks of agar 1 mm. square.

Potato-dextrose agar with hydrogen-ion concentrations in rather regular steps from P_H 3.0 to P_H 10.0+was used. The media were made as usual, except only one-half the required quantity of water was added at first. Equal quantities of the concentrated agar were placed in the flasks, each of which was plugged, properly labeled, and sterilized in the autoclave for 40 minutes at 8 pounds pressure. The quantities of acid or alkali necessary to bring the medium in

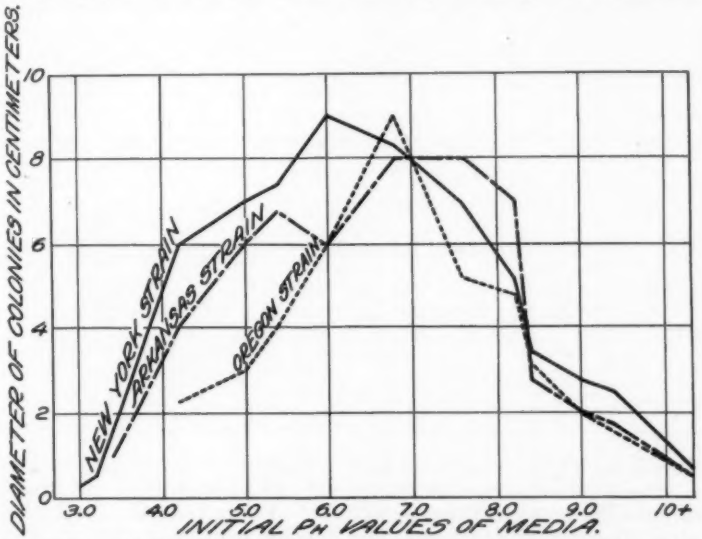


FIG. 4.—Influence of hydrogen-ion concentration of potato-dextrose agar on the growth of mycelium of *Ophiobolus graminis*. Data from Series 3, a–f, Table II

each flask to the desired reaction was calculated and the addition subsequently made. Calculated amounts of water were then added to the flasks to bring the medium in each to the proper dilution. This method allowed adjustment of media to various hydrogen-ion concentrations by the addition of either acid or alkali without materially affecting concentrations and ratios of the nutrients.

Equal quantities of the agar were then poured into Petri dishes, which were properly labelled. Sufficient agar remained in each of the stock flasks for hydrogen-ion determinations, which were made immediately at room temperature. Each agar-poured plate was inoculated as just described and then incubated under glass bell jars in diffused light at room temperature. At the end of seven days, at which time the largest colonies nearly covered the agar surfaces, the diameter of the colonies was measured. The resulting data are given in Table II, and the averages from Series 3, a–f, are shown graphically in Figure 4.

TABLE II.—*Influence of hydrogen-ion concentration of potato-dextrose agar on the growth of the mycelium of three strains of Ophiobolus graminis, as shown by measurements of diameter of colonies*

Initial P_H value of agar	Diameter of colonies in centimeters											
	Oregon strain						New York strain				Arkansas strain	
	Series 1a	Series 1b	Series 1c	Series 2a	Series 2b	Series 2c	Series 3a	Series 3b	Series 3c	Series 3d	Series 3e	Series 3f
3.0							0.0	0.0	0.0	0.5	0.0	0.0
3.2							.0	.0	.5	.5	.0	.0
3.4							.0	.0	2.0	1.0	1.0	1.0
3.8	0.5	0.5	0.5									
4.0	1.5	1.5	1.0									
4.2	2.0	2.5	3.0				2.0	2.5	6.0	6.0	4.5	3.5
4.4	3.0	3.5	3.5									
4.6	5.0	6.0	4.5	3.0	3.0	3.0						
5.0				5.5	6.5	6.5	3.0	3.0	7.0	7.0	.0	6.0
5.2	6.5		6.5									
5.4								4.0	8.0	7.0	7.0	7.0
5.6	7.0	7.0	7.0									
5.8	6.5		7.0	6.0	7.0	7.0						
6.0							6.0	6.0	9.0	9.0	6.0	6.0
6.2		6.0	6.0									
6.4	6.5	6.0										
6.6				7.0		5.5						
6.8							9.0	9.0	8.0	8.5		8.0
7.2				3.0	3.5							
7.4	4.0	4.0	3.0									
7.6							4.5	6.0	6.0	8.0		8.0
7.8	3.5	2.5	2.0									
8.0	1.5	2.0	1.5		2.5	2.5						
8.2	4.5	4.5	6.0				4.5	5.0	5.5	5.0	7.0	7.0
8.4	4.0	4.0	4.5	3.0	3.0	3.0	3.5	3.0	4.0	3.0	2.5	3.0
8.8	7.5	7.0	7.0	3.0	2.5	2.0						
9.0	3.5	2.0	4.0				2.0	2.0	3.0	2.5		2.0
9.2				3.0								
9.4								1.5	3.0	2.0	1.5	2.0
9.6				3.0	1.0	1.0						
10+				1.5			.5	.5	.5	1.0	.5	.5

It will readily be seen from these results that the three strains of the fungus varied somewhat in the amount of growth at the various hydrogen-ion concentrations. With the Oregon strain, the highest acidity allowing growth was P_H 3.8 in potato-dextrose agar. Growth increased rather regularly with decrease in acidity until a maximum was obtained at from P_H 5.6 to P_H 6.8. Beyond this, as the acidity diminished, mycelial growth of this strain decreased until it was checked at P_H 10.0+. The Arkansas strain exhibited growth at a concentration of P_H 3.4 and reached its maximum growth at P_H 6.8 to P_H 7.6. The most alkaline reaction permitting growth of this strain was just above P_H 10.0. The New York strain grew at all concentrations from P_H 3.0 to P_H 10.0+, with maximum growth at P_H 6.0.

In addition to the experiments with potato-dextrose agar, two experiments were conducted with the Oregon strain of the fungus in nutrient broth and Czapek's full nutrient solution. The results are given in Table III and represented graphically in Figure 5. In both, growth occurred at all reactions employed. However, the nutrient broth proved to be a more favorable medium at all hydrogen-ion concentrations than did the Czapek's full nutrient solution. In fact, the latter medium was found to be decidedly unfavorable for making these studies. Growth was comparatively feeble in the most

extremely acid and alkaline cultures, thus indicating that the concentrations necessary for inhibition lay slightly beyond the hydrogen-ion concentrations used in the experiment, which were P_H 4.8 to P_H 10.0+ for nutrient-broth growth and P_H 4.6 to P_H 10.0+ for Czapek's solution. In nutrient broth growth was best in cultures with an initial hydrogen-ion concentration of P_H 6.6. In Czapek's solution the greatest growth also occurred near this point. However, there was no striking optimum in the case of this medium. Final hydrogen-ion determinations of the media showed that all of the cultures involving Czapek's full nutrient solution became more acid with the growth of the fungus. The acid and slightly alkaline cultures of the nutrient broth shifted their reactions to greater alkalinity, while the most alkaline cultures tended to become slightly less alkaline. Unfortunately, no final determinations were made on the checks, so that it is impossible to say whether or not the latter changes were due to the growth of the organism or merely to changes in the reactions on standing.

TABLE III.—*Influence of hydrogen-ion concentration of nutrient broth and Czapek's solution on the growth of the mycelium of the Oregon strain of Ophiobolus graminis*

Nutrient broth				Czapek's solution			
Initial P_H	Final P_H	Growth	Average growth	Initial P_H	Final P_H	Growth	Average growth
		Gm	*Gm.*			*Gm.*	*Gm.*
4.8	5.4	0.033		4.6	4.2	0.018	
4.8	6.4	.045	0.039	4.6	4.2	.020	0.019
5.0	8.4	.100		6.0	5.4	.007	
5.0	8.4	.105	.102	6.0	5.4	.010	.0085
5.4	8.4	.090		6.4	5.8	.025	
5.4			.090	6.4	5.8	.020	.0225
6.6	8.6	.120		6.4	6.0	.030	
6.6	8.6	.120	.120	6.4	6.0	.035	.0325+
6.8	8.8	.095		6.4	6.2	.025	
6.8	8.6	.105	.100+	6.4	6.2	.035	.030
8.0	8.8	.085		7.8	7.6	.020	
8.0	8.8	.095	.090	7.8	7.8	.020	.020
10.0	8.8	.040		9.4	8.0	.020	
10.0	8.8	.055	.0475	9.4	8.0	.025	.0225
10+	8.8	.050		10+	6.4	.020	
10+	8.8	.070	.060	10+	6.0	.015	.0175
10++*	9.2	.025		10++	8.2	.015	
10++	9.2	.035	.030	10++	8.2	.020	.0175

* In the alkaline range, more NaOH was added to each succeeding flask; therefore, as one reads these figures, each 10+ had more chemical added than the previous one.

Although there are certain variations in the results in Table III, a few general conclusions may be drawn. The ranges of hydrogen-ion concentration in which *Ophiobolus graminis* will grow favorably are about the same for potato-dextrose agar, nutrient broth, and Czapek's solution. Growth took place in all cases where cultures possessed an alkaline reaction of or greater than P_H 10.0+, but it was very slight. This value, then, could be stated as approximately the most alkaline reaction in which vegetative growth of *Ophiobolus graminis* occurred. On the other hand, the most acid reaction permitting growth varied from P_H 3.0 and P_H 3.8, depending on the strain of the fungus. In these experiments the optimum hydrogen-ion concentration for the different strains varied too much to draw definite conclusions, except that if an average were taken it would occur on the acid side of neutrality.

These results vary somewhat from those obtained by Kirby (7). He obtained maximum growth of the New York strain on potato agar at a concentration of about P_H 9.0, and growth was inhibited at about P_H 3.2. The latter figure agrees very closely with the writer's results. However, the hydrogen-ion concentration for optimum growth obtained by Kirby is considerably more alkaline than that obtained in the present studies. Using corn-meal agar as a substratum he found the maximum growth at about P_H 9.2. The writer's cultures were kept in the light, while those studied by Kirby were kept in the dark. This may be a factor of importance. However, the writer is convinced that the strains of the fungus which he has been studying will grow better, in the media employed, at less alkaline reactions than those reported by Kirby. In this connection it may be said that Kirby (7) obtained less disease in wheat

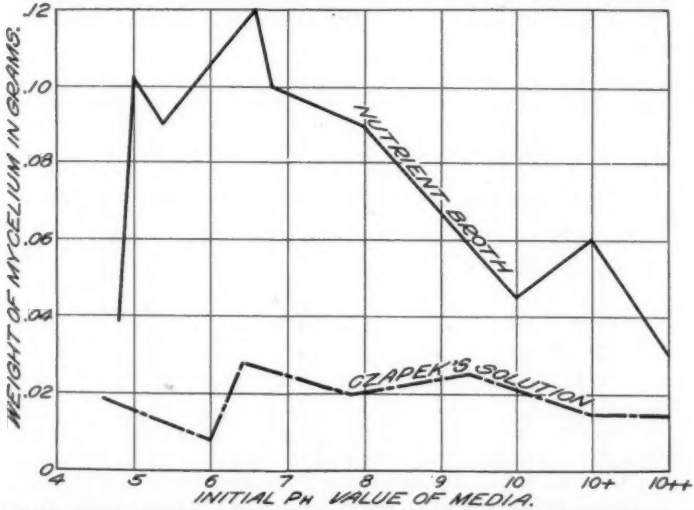


FIG. 5.—Influence of hydrogen-ion concentration of nutrient broth and Czapek's solution on the growth of the mycelium of *Ophiobolus graminis*. Data taken from Table III

plants when the soil in which they were growing was made acid by addition of certain fertilizers than when it was made alkaline, and likewise more disease was obtained in untreated soil than in limed soil. Kirby made no hydrogen-ion determinations on the soils treated in different ways; at least no data were published to this effect, so it is impossible to correlate the data for the growth of the organism in pure culture with those for the occurrence and intensity of the disease of plants growing in such soils. These were only preliminary studies, but it is evident that more work is necessary on this phase of the problem.

INFLUENCE OF LIGHT ON GROWTH AND SPORULATION OF THE FUNGUS

Cultures of *Ophiobolus graminis* in Petri dishes kept under glass bell jars in the diffuse light of the laboratory grew slightly less than cultures kept under darkened bell jars, as shown in Table IV. Al-

though the difference in the rate of growth of these two sets of cultures was only slight, it was generally constant. The variation in temperature between the two groups of cultures was never more than 1 degree, and as the studies on the influence of temperature on the rate of growth of the fungus show that differences of 1 degree or less make little difference in the rate of growth, it is concluded that the decrease in growth of the cultures placed in the light was due to the influence of light and not to that of temperature.

TABLE IV.—*Influence of light on the growth of mycelium of Ophiobolus graminis*

[Data based on 7-day-old cultures]

Strain of fungus	Agar medium used	In light or dark	Diameter of colonies in centimeters						
New York	Potato-dextrose	Light	8.0	8.0	8.0	8.0	8.0	8.0	8.0
	do	Dark	8.5	8.5	8.5	8.5	8.5	8.5	8.5
	String bean	Light	7.0	8.5	7.0	7.0	7.0	7.0	7.0
	do	Dark	7.0	7.5	7.5	7.5	7.5	7.5	7.5
Oregon	do	Light	4.0	4.5	4.0	4.0	4.0	4.5	4.0
	do	Dark	4.0	5.0	4.0	4.0	4.5	4.5	5.0
	do	Light	2.5	2.5	3.5	3.0	2.5	2.5	3.0
	do	Dark	3.0	4.0	3.5	3.0	3.0	3.5	4.0

The influence of light on sporulation was very marked in contrast to its influence on the growth of mycelium. With string-bean agar as a substratum, no culture kept in absolute darkness formed even a single perithecium, while most of those kept in the light formed fruiting bodies abundantly, as shown in Table V. One hundred and ninety-six cultures were observed at three different times and in all cases only mycelium was produced in those incubated in the dark. However, a few of the cultures kept in the light did not sporulate, which indicates that some influencing factor favorable for sporulation remains yet to be determined. The writer does not infer that perithecia will form only in the presence of light, for they occasionally are found on the roots of plants near the crown where they are formed in darkness, but from the laboratory experiments cited it is apparent that light favors the development of perithecia on the culture media used and under the conditions described.

TABLE V.—*Influence of light on the development of perithecia of Ophiobolus graminis*

[Data based on 47-day-old cultures]

Experiment No.	In light or dark	Total number of cultures	Number of cultures producing perithecia
1	Light	20	14
	Dark	20	0
2	Light	60	46
	Dark	40	0
3	Light	36	30
	Dark	20	0

PATHOGENICITY OF THE FUNGUS

All three strains of the fungus used in these studies were pathogenic on wheat plants. To prove this, disinfected Goldcoin wheat was sown in soil that had been disinfected by steam and subsequently

inoculated with the different strains of *Ophiobolus graminis*. The soil used was a fine silt loam, as previously described, and was placed in an autoclave for 2 hours under 15 pounds steam pressure. Equal amounts of this soil were placed in water-tight metal cans 6 inches in diameter and 9½ inches deep. The upper 3 inches of soil was then removed from each can, and kept separate. To each portion of this soil was added 100 gms. of inoculum, which consisted of the fungus growing on cooked oat and barley kernels, as described previously. Each portion of soil and inoculum was then mixed together very thoroughly and placed in the proper can. The control cans were similarly filled with their portions of soil and 100 gms. of the uninoculated oats and barley medium. The wheat seed was sown 1½ inches deep in this uniformly inoculated layer. After sowing, all the soil cans were immediately placed in constant-temperature water baths in a greenhouse. Samples of the seed used in the experiment were disinfected with 1 to 1,000 mercuric chloride, washed in sterile water, and then placed on agar in Petri dishes. In all cases the kernels were found to be free from seed-borne fungi.

The plants growing in the inoculated soil soon became diseased, and later many of them died. In all cases the check plants remained healthy and the roots were white and disease-free, as indicated in Table VI and Plate 4. Microscopic examination showed the presence of the fungus in the tissues of the infected plants, and a pure culture of the organism was isolated from the attacked seedlings in the early stages of the disease. Dark-brown lesions developed on the roots and culms of all of the infected plants, and many of the leaves turned yellow and died. Several experiments similar to the above were performed later in the course of other studies, and similar results were obtained. All three strains of the fungus were found to be readily pathogenic, but the New York strain was more virulent and killed a greater percentage of plants than either of the other strains. This was possibly due to the fact that the New York strain fruited rather freely, making it possible to use ascospore cultures for transfers.

TABLE VI.—*Pathogenicity of Ophiobolus graminis on Goldcoin wheat grown in steamed soil, inoculated with pure cultures of the parasite*

[The controls were uninoculated]

Strain of fungus	Number of plants	Condition of plants [a]
New York	150	All plants diseased.
Control	50	No disease.
Oregon	150	All plants diseased.
Control	50	No disease.
Arkansas	150	All plants diseased.
Control	50	No disease.

[a] The disease was most severe in the pots inoculated with the New York strain. Plants grown in the pots inoculated with the Oregon strain showed a less severe attack, but they were more severly diseased than the plants grown in the pots inoculated with the Arkansas strain of the parasite.

It was also proved, under aseptic conditions, that *Ophiobolus graminis* caused the take-all disease of wheat. Wheat plants were grown on Crone's agar in glass culture tubes 2½ inches in diameter

and 20 inches long and inoculated with the fungus. Prior to planting and inoculating, the tubes containing the medium were plugged with cotton and sterilized at 15 pounds pressure for 40 minutes. After the agar solidified, disinfected Goldcoin wheat seeds which had been previously germinated for 5 days on potato agar in Petri dishes were planted in the large tubes. The previous germination was carried out to insure seedlings that were free from fungi and bacteria. The seed was handled under aseptic conditions throughout the whole process previous to inoculation. Two days after planting the seed, a block of agar containing mycelium of a single strain of *O. graminis* was inserted in each tube, except the control tubes, which were handled similarly but not inoculated. All of the tubes were placed in the laboratory in such a position that they received equal illumination, and the plants were carefully watched for any indication of disease.

The fungus soon spread over the surface of the agar in the inoculated tubes, and in 4 days symptoms of the disease on the wheat seedlings began to appear. The characteristic dark lesions associated with take-all first started at or near the base of the roots. This discoloration then spread both up on the culm and farther down on the roots, and the leaves soon turned yellow and died. By the time the plants were 28 days old, all of the inoculated plants had died, while the uninoculated plants were still healthy and thriving. These latter had grown to the top of the tubes and were badly crumpled, due to their upward elongation being stopped by the plugs in the mouths of the tubes. A week later part of them had died, apparently due to lack of space and the drying out of the agar, but no disease lesions were visible. The results are given in Table VII.

TABLE VII.—*Pathogenicity of Ophiobolus graminis on Goldcoin wheat grown under aseptic conditions on agar contained in culture tubes*

[Inoculations were made with pure cultures of the parasite. The controls were uninoculated]

Strain of fungus	Number of tubes	Number of plants in each tube	Total number of plants dead in all the tubes	
			After 21 days	After 28 days
New York	4	3	12	12
Control	2	3	0	0
Oregon	4	3	0	12
Control	2	3	0	0
Arkansas	4	3	0	12
Control	2	3	0	0

Pure cultures of *Ophiobolus graminis* were obtained by putting bits of the culms of the infected plants grown in the culture tubes on fresh agar in Petri dishes. Plants from uncontaminated culture tubes invariably gave a pure culture of the parasite.

Delacroix (*3*) inoculated soil with a water suspension of ascospores of *Ophiobolus graminis* obtained from perithecia which had developed on diseased plants in the field. Wheat plants grown in this inoculated soil developed a rather high percentage of infection and

showed marked injury. Plants grown in the same manner and under the same conditions in uninoculated soil remained healthy.

Waters (*15*) found that pure cultures of this organism were pathogenic on wheat plants growing in tubes of sterilized soil to which a pure culture of *Ophiobolus graminis* had been added. Kirby (*7*) also found that wheat plants developed the characteristic symptoms of take-all when grown in soil inoculated with pure cultures of this fungus.

OVERWINTERING OF THE PARASITE

These studies were carried out with three kinds of material: (1) agar cultures of the parasite in ordinary test tubes; (2) artificially inoculated soil; and (3) diseased plant material. All of the experiments were conducted during the winter of 1922–23, which was unusually severe at Madison, Wis.

PURE-CULTURE STUDIES

All three strains of the parasite, as used in previous experiments, were cultured on potato-dextrose agar in test tubes. After a good growth of mycelium was obtained the cultures were placed outdoors in an exposed position throughout the winter. In the spring it was found that several tubes had burst, due to the freezing of the agar. Transfers of the mycelium from all of the overwintered tubes showed that all of the cultures were alive. In all cases vigorous cultures of the three strains were obtained.

STUDIES WITH ARTIFICIALLY INOCULATED SOIL

An experiment was conducted with three lots of soil contained in 6-inch metal cans. This soil was inoculated on October 18, 1922, with the mycelium of the New York, Arkansas, and Oregon strains of the parasite. Goldcoin wheat seed was planted at the time of inoculation. The plants showed signs of disease on November 22, 1922, and at this time they were removed from the soil as completely as possible, including the roots, and the soil was left out of doors throughout the winter. All of the plants removed from the soil were examined carefully for perithecia, but none were found. On March 26, 1923, Goldcoin wheat seed was again planted in all the cans. Early in May take-all developed in all of the 21 plants growing in the soil which had been inoculated with the Oregon strain of the parasite. The disease did not develop in plants growing in the soil inoculated with the New York or Arkansas strains and the disease was mild in the plants growing in the soil inoculated with the Oregon strain.

A second experiment was conducted in exactly the same manner and at the same time as the one just described, except that no seed was planted in the soil in the fall after inoculation. In the spring Goldcoin wheat seed was sown, and a severe case of take-all developed in all of the 21 plants growing in the soil which had been inoculated with the Arkansas strain of the parasite. No disease occurred in soil inoculated with the Oregon or New York strains of the fungus, and more occurred in the uninoculated controls in either experiment. It is not known just why certain strains of the fungus were killed during the winter. Possibly unevenness of snow cover may have been the cause.

A third experiment was carried out on a small plot of sterilized soil. This was divided into three parts, and each part was inoculated in the fall of 1922 with one of the strains of the parasite. Goldcoin wheat seed was sown in this soil in the fall and about 400 plants developed to the seedling stage, when all but 57 became diseased and died in the fall. Fifteen plants of those living were in the soil inoculated with the New York strain of the fungus, 28 in that inoculated with the Oregon strain, and 14 in that inoculated with the Arkansas strain. Plants growing in uninoculated control soil grew thriftily until cold weather came on. In the spring only 16 plants remained alive in the plots which had been inoculated, and these plants showed no signs of disease. All of the plants in the uninoculated control plot were winterkilled. Owing to the unoccupied space in the inoculated and uninoculated soils in the spring, it was decided to plant Marquis seed in both plots to determine whether the fungus was still living in the soil and pathogenic. This was done on April 27, and the 16 Goldcoin plants were allowed to remain in the inoculated plot. By May 17 all of the Marquis plants growing in the last-mentioned plot commenced to turn yellow and soon developed a severe case of typical take-all seedling blight. About 95 per cent of these plants died in a short time, while the plants growing in the uninoculated control soil developed normally.

The 16 overwintered Goldcoin wheat plants growing in the infested soil showed no signs of disease until the last of May, when they turned yellow and died. It is believed that the winterkilling of the Goldcoin control plants and the escape of the plants growing in the infested soil is explained on the basis of irregularities in snow drifting during the winter.

STUDIES WITH INFECTED PLANT TISSUE

Plant material obtained from the experiments dealing with host susceptibility to the New York strain of the parasite were employed in this study on overwintering. In one case five No. 00 ash pails (5 gallons capacity) containing infected stubble in infested soil were left outdoors over winter. This stubble was rooted in the soil, and the soil was not disturbed, except when stubble was removed for purposes of making examinations for perithecia. In another case the infected stubble was removed from the soil and placed in open earthen flowerpots. In both cases the material without protection was exposed to the winter weather.

Examinations were made on October 17, 1922, and perithecia were forming on the culms just above the crown in both lots of stubble. By January 3, 1923, asci had developed and many contained immature spores. Numerous paraphyses were also present. By April 1 the ascospores appeared mature, and those from one plant were tested for germination. It was found that 4.5 per cent of the ascospores germinated when placed in distilled water on a slide under a cover glass. The same plant with the remaining fruiting bodies was then placed outside in its previous location, and on May 1 this plant was brought into the laboratory with several others, and ascospores from all of them were tested for their viability as before. Spores from the plant previously tested still showed about the same percentage of germination, while spores from other plants gave practically 100 per cent germination. Some of these spores were obtained from fruiting

bodies which were produced 2 to 3 cm. above the soil line on plants still standing as in the soil, while others were from plants frozen in the bottom of the flowerpots where they had been placed.

From the foregoing evidence it is clear that *Ophiobolus graminis* will survive the winter in the mycelial and ascospore stages. Although there were cases when the fungus was killed during the winter, it is evident that, under certain conditions at least, the fungus can live through rather severe winter conditions.

AGE OF WHEAT PLANTS IN RELATION TO SUSCEPTIBILITY

In order to obtain data on the relation of age of wheat plants to susceptibility to *Ophiobolus graminis*, it was necessary to sow all of the seed used in any one experiment at one time and to apply the parasite at intervals throughout the development of the plants, or to plant the seed at intervals and inoculate the plants at one time. Both methods were used with satisfactory results. In this study four experiments (numbered I to IV) were conducted.

Experiment I was conducted out of doors in soil contained in No. 00 ash pails of 5 gallons capacity. Experiments II, III, and IV were performed in the greenhouse in Wisconsin soil-temperature-control tanks, and the temperature of the soil was held as near 18° C. as possible throughout the growth of the plants. In all of the experiments the water content of the soil was 55 per cent of the total water-holding capacity, and this percentage was maintained as constant as possible throughout the experiments by frequent weighings. An excess amount of seed was always planted in the containers, and after the plants had reached about the third-leaf stage they were thinned to a uniform number. The inoculum used in these experiments consisted of the mycelium of the New York strain of the parasite growing on the barley and oat-kernel medium previously described.

In the series sown at one time the first inoculation was made when the seed was planted. In the series sown at intervals the whole series was inoculated at the time the last planting was made. Growing plants were inoculated by carefully removing the upper 2 inches of soil from the pots, great care being taken not to cause undue injury to the roots. The mycelium of the parasite growing on the barley and oat-kernel medium was then added to the soil, the whole mass was well mixed and returned to the pots and carefully worked around the roots. Great care was taken to use cultures showing the same amount of growth and to add the same amount of medium to all the pots inoculated. In the control pots, in each case, an equivalent amount of sterile barley and oat medium was added to the soil in the same manner.

In Experiments I and II, California Club wheat was used, and the seed was sown at one time. The inoculations were made at intervals of 2 weeks after sowing, the first inoculation being made in one set at the time of sowing. As shown in Table VIII, all of the inoculated plants in these two experiments developed take-all and all eventually succumbed. All of the plants grown in the uninoculated soil remained healthy and matured normally. The amount of infection produced in one of these experiments is shown in Plate 5. The plants from seed which was inoculated at the time of planting began to turn yellow soon after emergence, and most of them died while in the seedling stage.

TABLE VIII.—*Period of susceptibility of California Club wheat plants to the attack by Ophiobolus graminis in Experiments I and II*

[The seed was sown at one time and the inoculum was applied at intervals during the growth of the plants. Controls were uninoculated]

Number of days after planting before inoculum or media was added	Treatment of plants	Number of diseased plants					
		Experiment I (out of doors)			Experiment II (in greenhouse)		
		Number of plants	Number of plants badly diseased	Number of plants slightly diseased	Number of plants	Number of plants badly diseased	Number of plants slightly diseased
Same day	Inoculated	20	20	0	7	7	0
Do	Control	10	0	0	7	0	0
14	Inoculated	20	0	20	7	0	7
14	Control	10	0	0	7	0	0
28	Inoculated	20	0	20	7	0	0
28	Control	10	0	0	7	0	7
42	Inoculated	20	0	20	(a)		
42	Control	10	0	0	7	0	0
56	Inoculated	20	0	20	7	0	7
56	Control	10	0	0	7	0	0
70	Inoculated	[b] 20	0	20			
70	Control	10	0	0			

[a] Plants destroyed by rats. [b] Plants were in bloom.

In Experiments III and IV, Goldcoin seed was sown at intervals of two weeks. The inoculations were made at the time of the last planting. As in Experiments I and II, all the inoculated plants became diseased, while the control plants remained healthy, as indicated in Table IX. The plants from seed sown at the time of inoculation developed the disease first and showed the most severe injury. Many of them died in the seedling stage, and none of them matured seed. Likewise the plants that were 2 weeks old when inoculated were attacked in a similar manner, but to a less degree. However, the plants that were older when inoculated did not show the effects of the disease until later.

TABLE IX.—*Period of susceptibility of Goldcoin wheat to attack by Ophiobolus graminis in Experiments III and IV*

[Plants grown under greenhouse conditions, in soil held near 18° C. The seed was sown at intervals and the inoculations were made at the time of the last planting. Controls were not inoculated]

Age of plants when inoculum or media was added (days)	Treatment of plants	Number of diseased plants					
		Experiment III			Experiment IV		
		Plants grown from June 27 to Sept. 12			Plants grown from Sept. 7 to Dec. 6		
		Number of plants	Number of plants badly diseased	Number of plants slightly diseased	Number of plants	Number of plants badly diseased	Number of plants slightly diseased
Just planted	Inoculated	30	0	0	30	30	0
Do	Control	10	0	0	10	0	0
14	Inoculated	30	30	30	30	15	15
14	Control	10	0	0	10	0	0
28	Inoculated	30	30	30	30	0	30
28	Control	10	0	0	10	0	0
42	Inoculated				30	0	30
42	Control				10	0	0

That all of the inoculated plants of the four experiments became diseased, regardless of their age at the time of inoculation, is very significant. In two of the experiments part of the plants were in the flowering stage at the time of their inoculation, but these plants showed distinct lesions produced by the fungus, and by October Ophiobolus perithecia had formed on them. The same was true with the plants inoculated earlier, except that these were attacked more severely.

From these results it is evident that the varieties of wheat studied are susceptible to attack by the fungus during their entire growing period. The disease is most severe during the seedling stage of the host, and only a small portion of the plants attacked at this stage ever recovered. Whether, under field conditions, they would recover sufficiently to produce a crop is not known, but reports from field observations make this seem improbable (*13*, *9*). Plants infected during the late stages of development, or plants recovering from earlier infections, produce symptoms known as "white heads."(*1*)

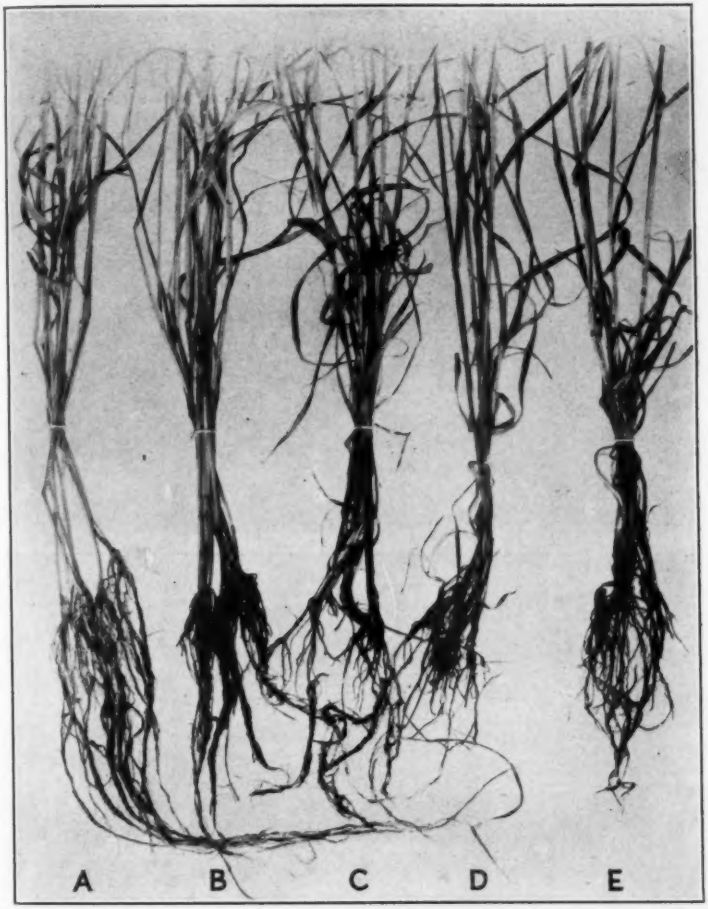
Although the inoculation method used in these experiments caused considerable root mutilation, it is believed that this does not seriously affect the results obtained. This is supported by the fact that the most severe cases of disease always occurred in the plants grown in the soil which was inoculated at the time of sowing the seed. Furthermore, results discussed later show that the fungus hyphae readily penetrate the unbroken epidermis of the roots.

PARTS OF WHEAT PLANT SUSCEPTIBLE

Preliminary studies to determine what parts of the wheat plant are susceptible to attacks of *Ophiobolus graminis* were carried on in large glass tubes containing cultures of the fungus. When the surface of the medium was thoroughly covered with mycelium, a 4-inch layer of cooled Crone's plant agar was poured over the colony. Wheat seedlings, previously germinated under sterile conditions, were then placed in the tubes, and the cultures were placed in a light place in the laboratory. Plants for experimental controls were grown in the same manner, except that no fungus was in the tubes.

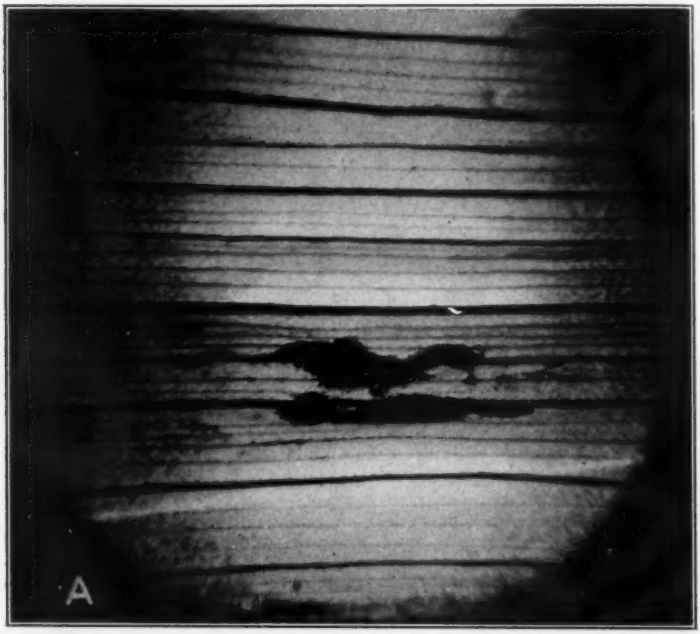
The roots of the seedlings were the first parts to be attacked. In from two to three days after planting, dark lesions began to show on the roots, and soon the entire roots were blackened. The culms did not show any signs of attack until four to five days after the roots had shown symptoms of invasion. The control plants remained entirely healthy.

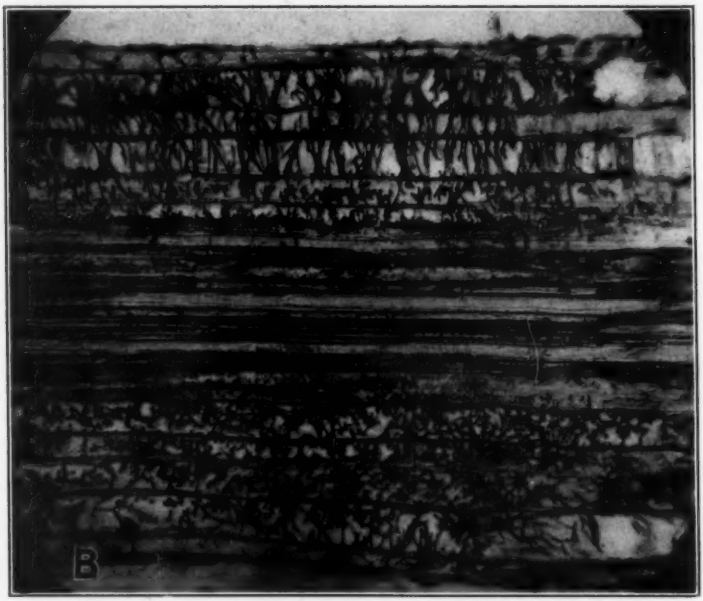
In studying the histology of diseased tissues, as reported later, the fungus was found to enter the roots at any point when the plants were grown in the soil. Mycelial threads could be seen entering through the epidermis at various points along young roots, except near the distal ends where they were elongating rapidly. Single lesions also were noted on roots that had healthy tissues for several centimeters above and below the diseased portion. These observations, together with the fact that the roots could be infected while the rest of the plant was free from the fungus, definitely prove that the roots are susceptible to attack by *Ophiobolus graminis*.



Healthy (A) and diseased (B, C, D, E) California Club wheat plants, 89 days old, grown in the greenhouse. The soil in which the diseased plants were grown was sterilized and then inoculated with mycelium of the New York strain of *Ophiobolus graminis*

A.—Healthy plants grown in sterilized uninoculated soil
B.—Diseased plants grown in sterilized soil inoculated at the time of sowing
C.—Same as B, except not inoculated until 14 days after sowing
D.—Same as B, except inoculated 28 days after sowing
E.—Same as B, except inoculated 56 days after sowing





A.—Photomicrograph of an isolated lesion on the sheath of a Goldcoin wheat plant artificially inoculated and grown in the greenhouse. × 16

B.—Photomicrograph of a longitudinal section of a wheat root, artificially inoculated and grown in the greenhouse, showing the cells of the cortex filled with the mycelium of *Ophiobolus graminis*. × 160

An experiment similar to the one just described was conducted on the culms. For this purpose the plants were grown in tubes containing Crone's agar. When the plants were about 4 inches tall, cooled potato-dextrose agar was poured into the tubes to a depth of one-half inch. Ophiobolus was placed in some of these tubes, and others were left uninoculated for controls. The culms began to turn dark at the surface of the agar soon after the mycelium had spread over the medium and had come in contact with the culm tissues. The darkening progressed up and down the culm, and as the agar dried away from the surface of the walls of the tubes, the fungus grew down through this opening and attacked the roots. The fungus spread through the agar to a very slight extent. As in the case of the roots, it was found that the fungus penetrates the leaf sheaths and culms at any point below the surface of the soil. Often isolated lesions were noted on the sheaths, thus indicating that the fungus had entered at that point (pl. 6, A).

The green leaves of 10 plants in the early heading stage growing in the field also were tested for susceptibility to infection. Rubber rings, with a diameter a little less than the width of the leaves, were placed on the surface. A water suspension of ascospores was placed in these and a cover glass placed over each ring. The rings were held in place on the leaves with fine spring clamps. No infection took place by this method of inoculation. A few of the spores sent out short germ tubes, but they never entered the leaf. This method seemed unsatisfactory and it was discontinued because the water evaporated too rapidly and left the germinating spores dry.

Numerous wheat heads grown in the field were dipped in an ascospore suspension and then covered with glassine bags. The bags were tied closely about the culm to prevent evaporation, and were allowed to remain in place for four days, after which they were removed. No sign of disease developed, and the heads matured good seed. Ten plants growing in pails in the greenhouse were thoroughly covered with a spray of ascospores and placed in a moist chamber. Part of the plants used were young, while others had headed out. The air of the chamber was kept saturated with moisture, and the temperature was held as near 20° C. as possible. The light conditions in the chamber were poor. After four days the plants were taken out of the chamber and placed in the greenhouse. No visible signs of disease appeared on the plants. Microscopical examinations showed that a number of the spores had germinated, but in no case had they penetrated the wheat plants. The plants were examined the day after they were removed from the moist chamber and at short intervals for several weeks, but no sign of disease appeared. Finally, bits of mycelium were placed on leaves and heads of 10 more plants. A needle was run through the masses of mycelium and into the tissues of the plant. The plants were placed in a moist chamber for four days under the same conditions as before. They were allowed to stand one day after being removed before being examined for infection. By that time the mycelium had dried up and none could be found in the tissues, except a few pieces pushed in with the needle. Later no mycelium could be found, and disease symptoms never developed.

From the results obtained in these experiments, indications are that the leaves and heads are not susceptible to attack by *Ophiobolus*

graminis. The writer feels fairly confident that the leaves and heads of wheat plants growing in the open field are seldom if ever attacked. The dying of the upper parts of the plant evidently is due either to the inability of the roots and culms to allow sufficient food materials and water to reach these parts or to the development of toxic substances in the regions of infection. The first view is the one which has been generally held for this type of phenomenon, but data are now available which indicate that toxic substances are given off by some fungi, and these toxins are capable of causing plants to wilt (*4*). Possibly both of these factors operate, but the former would appear to be the chief factor in this case, due to the great reduction of the root area and the partial clogging of the vessels.

HISTOLOGY OF DISEASED WHEAT PLANTS

Suitable material for the histological studies was obtained by killing and fixing diseased and healthy plants each day as the disease progressed. Virgin soil was thoroughly sterilized by steam pressure, then Ophiobolus mycelium, previously grown on cooked cereals, was thoroughly mixed with the soil. This was placed in small cans, and disinfected Goldcoin wheat seed was planted at a depth of 1½ inches. An equal number of uninoculated cans for growing control plants were also prepared in the same manner, except that no fungus was added to the soil. The cans were placed in constant-temperature tanks and the temperature of the soil was maintained at 12° C. The plants from an inoculated and an uninoculated can were carefully washed with water and the plants killed, embedded in paraffin, and later sectioned according to the usual technique. Various killing and fixing agents, as Flemming's medium, Gilson's, Carnoy's, and chrom-acetic solutions, were used. The material killed and fixed in Gilson's fluid gave the best results for general histology, but such material was very poor for showing the detailed structure of the host cells. Two series of such plants grown at separate times were prepared in the above manner.

While pursuing these studies the macroscopic symptoms also were carefully noted. The first primary root broke through the coleorhiza two days after planting and three days later this root appeared slightly dark and watersoaked near its base. The next day this water-soaked area had turned a dark brown to black, and on the eighth or ninth day after planting the primary roots had become so badly decayed that they easily broke off. In the meantime, as the other roots developed they were also attacked, and lesions developed which were similar to those occurring on the first primary root. Often a root showed several distinct and separate lesions, indicating that the fungus had penetrated at various points. The coleoptile emerged from the soil on the eleventh day and an examination of the lower part of this, next to the old seed, revealed a slight discoloration. The following day this portion of the seedling was dark brown, and the lesion was extending farther up.

On the fourteenth day after planting the seedlings began to show symptoms just above the ground line. Brown streaks were visible on the coleoptile near the soil, but they did not extend up the sheath. The infected plants were decidedly smaller than the control plants

and at the second-leaf stage (15 days old) the first leaf began to turn yellow. Some plants showed this chlorotic condition before the first leaf appeared, but these were only occasional cases. The bright-green color of the leaf faded out, leaving a yellow color in its place. At times this area turned bronze instead of yellow. The discoloration usually started first at the tip of the leaf and extended down the blade, but it often started back of the tip or on one edge. Finally, the whole leaf yellowed and died and turned a light-brown color. In a very short time the second leaf also became discolored similarly to the first one and died. Some of the infected plants recovered and lived indefinitely. Usually the primary root system on these plants was gone and part of the permanent roots was badly decayed, but new ones were formed to replace them. In case the plant had sufficient vitality to form new roots as fast as the fungus killed the old ones it lived, but if its vitality was low and new roots were not formed it soon died. In this event the roots became entirely decayed and the culm turned a dull-black color for about a centimeter above the soil line.

The embedded material for the histological studies was sectioned 5 to 10 microns in thickness and stained in saffranin and light green. These stains proved satisfactory and enabled the writer to follow the fungus fairly readily. Often the mycelium grew along the surface of the tissues and formed a mat of hyphae before penetrating the cells. The mycelium penetrated the wall at any point. Occasionally there was a swelling of the hyphae on each side of the wall, with constrictions of the segments in the wall.

The fungus was found to enter the plant at any point below the surface of the soil. The coleorhiza was the first tissue found invaded, and the parasite often spread from this point into the developing root. However, the primary infection of the seedling took place chiefly by the fungus growing between the coleorhiza and the root, with subsequent penetration. As the root elongated, it became invaded at points below the primary infection, and in case the basal parts escaped infection, as they often did, the root was usually attacked at other points. After the fungus had entered the root it spread in all directions. However, usually the fungus did not spread lengthwise as much as it did toward the center of the root, as shown in Plate 6, B, because the root was being penetrated at various points along its entire length. On reaching the endodermis of the young root, the fungus stopped its inward growth and did not penetrate this until most of the cells of the cortex had been killed. Even after penetrating the central cylinder the fungus appeared to remain, for the most part, in the parenchymatous cells, and was found inside the bundles of only the old and very badly diseased plants.

The fungus invaded the leaf sheaths and culms in the same manner as the roots, penetrating at all points below the surface of the soil. The fungus was found in all the tissues of this region except the primordia. Frequently the coarse type of mycelium was found in the vessels of the lower portion of plants inoculated in large test tubes.

Badly infected plants are usually black at the base. This blackening may occur under the outer leaf sheath or it may show through

this sheath. Usually the blackening is due to a staining of the tissue by the invading fungus, or it may be due to the crusts of the mycelium.

Examinations show that the cytoplasm and nucleus begin to disintegrate as the fungus enters a cell. In fact, the nucleus was often seen breaking up before the fungus actually entered the cell, and by the time the hyphae were well distributed through the cells no sign of the nucleus could be seen. The cell wall was also affected by the presence of the fungus, but the exact nature of the change was not determined. On staining the tissues with saffranin and light green, the cellulose cell walls took up the light-green stain until the fungus entered the cell, or the adjoining cell, then the walls took up and retained the saffranin. It appears from this that enzymes or toxic substances penetrate the host cells in advance of the fungus. The fact that the cell contents of the host showed disintegration before any hyphae entered the cell also substantiates this view.

SUMMARY

The name *Ophiobolus graminis* Sacc. is used in this paper to desinate the fungus causing the take-all disease of wheat.

Perithecia of the New York strain of this parasite were formed in pure cultures on artificial media and on wheat plants growing in soil that had been inoculated with the fungus. Ascospores and paraphyses were formed in abundance.

When conditions were favorable the ascospores germinated rapidly, sending out from 1 to 3 germ tubes which branched and rebranched, forming a network of hyphae. Numerous cultures of the New York strain, made from single ascospores, produced perithecia and mature ascospores, thus showing that this strain of the fungus is homothallic. The Oregon and Arkansas strains studied were never induced to sporulate.

Although the three strains of the parasite reacted similarly to temperature, they showed some variation in this regard. In all cases growth took place between 4° and 33° C., but it was very slight at the extremes. The optimum temperature for the growth of the New York strain was found to range between 19° and 24°, whereas the optimum for the Oregon and Arkansas strains was between 23° and 24°.

Light checked mycelial growth slightly and greatly stimulated sporulation in the New York strain of the parasite.

The strains of the parasite used in these studies grew within the P_H range of 3.0 to 10+. The optimum hydrogen-ion concentration for the New York strain was found to be P_H 6.0 and that of the Oregon strain was P_H 6.8. The optimum for the Arkansas strain was less limited, in that it extended between P_H 6.8 and 7.6.

The ascospores and probably the mycelium of the parasite were found to overwinter in plant refuse in the soil. Mycelium overwintered readily in agar cultures in test tubes.

The varieties of wheat used in these investigations (Goldcoin, California Club, and Marquis) were all found to be susceptible to infection by *Ophiobolus graminis* during the entire growing period of the plants. All of the studies made thus far show, however, that plants are most susceptible in the seedling stage.

Infection was never obtained in any part of the plants much above the soil line.

Histological studies were made from plants exhibiting various stages of infection and these showed that the parasite entered the unbroken epidermis of the underground portions of the leaf sheaths, culms, and roots. The parasite first destroys the cortex of the roots and later enters the central cylinder. It also destroys the leaf-sheath and culm tissues and later enters the vessels, but it does not appear to make much progress after it enters the vessels.

These studies indicate that the bleaching of the aboveground portions of plants is not due to a systemic infection of the whole plant, but to the cutting off of the food materials and water supply and possibly to the toxic substances.

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INFLUENCE OF SOIL TEMPERATURE AND MOISTURE ON INFECTION OF YOUNG WHEAT PLANTS BY OPHIOBOLUS GRAMINIS[1]

By H. H. McKinney, *Pathologist*, and R. J. Davis, *formerly Field Assistant*, *Office of Cereal Investigations, Bureau of Plant Industry, United States Department of Agriculture.*[2]

INTRODUCTION

There are many statements in the literature regarding the influence of weather and soil drainage on the occurrence of the take-all and foot-rot diseases of wheat. In most cases, however, these reports have been based on rather casual observations which, in many instances, have been made on the foot rots in general rather than on take-all, the specific foot rot caused by *Ophiobolus graminis*. Doubtless the lack of agreement among certain workers as to the influence of temperature and moisture on the take-all disease is due largely to the fact that they have been dealing not with one disease, but with several which are very similar in appearance but caused by different parasites.

Gaillot (*6*)[3] states that in France wheat foot rot is favored by mild, moist weather, whereas Reuther (*13*) states that in Germany the foot disease was favored in 1913 by vernal frosts. In neither of these reports is it clear which foot rot is referred to, but it appears that these workers have in mind the foot rots as a whole. According to Lindau (*10, p. 256*) wet soil favors infection by *Ophiobolus graminis*, and directly in line with this Dombrovski (*5*) states that the proper drainage of the soil reduces losses due to this parasite.

McAlpine (*11*) states that opinions are most conflicting in connection with the occurrence of take-all in Australia. He states that some observers claim it to be more prevalent during wet seasons, while others say that dry seasons favor the disease. His own observations indicate that the disease occurs under all climatic conditions. Robinson (*14*), also working in Australia, states that a dry summer followed by a wet winter affords the most favorable conditions for the development of the disease. Sutton (*16*), working in western Australia, and Waters (*17*), working in New Zealand, claim that wet soil conditions favor take-all, whereas Hori (*7*), working in Japan, thinks the disease is less prevalent on poorly drained soils. In this connection, however, it should be pointed out that Hori's observations included a large number of cases of take-all on barley. It is entirely possible that the disease on this host may not react the same as on the wheat plant when placed under similar soil conditions.

In this country take-all has been observed for so short a time that little definite information is at hand concerning its development in the field under different temperature and moisture conditions. Kirby (*9*), working in New York, states that his observations indicate little or no difference in the amount of infection occurring on high

[1] Received for publication Dec. 19, 1924; issued January, 1925.

[2] These investigations were carried on in cooperation with the Wisconsin Agricultural Experiment Station.

[3] Reference is made by number (italics) to "Literature cited," p. 840.

and on low ground. He states, however, that in a few fields the infection appeared to be very much heavier on the lower and wetter land. Rosen and Elliott (*15*), working in Arkansas, also state that the disease seems to be favored by wet soil.

One of the writers (McKinney) has observed take-all under many different field conditions, and while the disease has appeared to be more severe in low undrained portions of some fields, this has not always been the case. Obviously such apparent irregularities emphasized the necessity for studying the influence of temperature and moisture under controlled conditions, and, accordingly, a series of such experiments was planned and carried out, the results of which are presented herewith.

EXPERIMENTAL METHODS

All of these studies were conducted under greenhouse conditions in the soil-temperature tanks and in controlled-temperature chambers of the Wisconsin Agricultural Experiment Station, at Madison. The soil-temperature apparatus and the general methods employed in these studies were identical with those used in the studies on the Helminthosporium disease (*12*). The temperature chambers have been described by Dickson (*4*) and it is unnecessary to discuss them in this paper.

The soil used in these studies was a fertile loam obtained from a wood lot near Madison. Although this was virgin soil, it was infested with several grass parasites which attack wheat, and it was necessary to disinfect all of it. Usually the soil was subjected to live steam at about 1 pound pressure for four hours. In other cases it was heated for one hour at 10 to 15 pounds pressure. Both methods gave good results and produced no toxic effect on the plants. One lot of this soil contained somewhat more organic matter than the other, as indicated by their moisture-holding capacities of 67 per cent and 53 per cent, respectively.

The various soil moistures employed in any one experiment were obtained by careful weighings and moisture adjustments before putting the soil in the temperature-tank soil containers. All soil moistures were calculated on the basis of the water-holding capacity of the soil and the moisture content of the soil was maintained as nearly constant as practicable throughout the experiments. The pots were weighed each day and water was added to replace that lost.

The wheat seed used in all of the experiments was of the Goldcoin variety generally known as Junior No. 6. All seed was disinfected in a 1:1,000 solution of mercuric chloride for 10 minutes, then thoroughly washed in running water before planting.

The parasites used for inoculation were obtained from Oregon and New York. The Oregon strain was isolated by Hurley Fellows from wheat collected near Corvallis, Oreg., in 1921, by A. G. Johnson, H. P. Barss, and M. B. McKay. The New York strain was kindly supplied by R. S. Kirby. Both strains originated from single ascospores, and they maintained their pathogenicity throughout the work. The virulence of the Oregon strain seemed to become reduced toward the end of the studies, and the New York strain was used in its place. The loss of virulence of the Oregon strain may have been due to the fact that it never sporulated, and consequently all in-

creases had to be made from mycelium. The New York strain produced ascospores rather abundantly at times and these were used for making new cultures.

The parasites were cultured in Erlenmeyer flasks, on a mixture of equal portions of barley and oats which had been thoroughly cooked and sterilized. The fungus increased rapidly on this medium, and the medium did not produce a toxic effect on the wheat plants when it was added to the soil, as was the case when cooked wheat kernels were used. Inoculations were made by adding to the sterilized soil a given weight of the barley-oat medium on which the fungus was growing. This was accomplished by first removing the upper 3 inches of soil from the containers and thoroughly mixing the inoculum with it. The inoculated soil was then returned to the pots and the seed was sown immediately. An amount of uninoculated medium equivalent to the amount added to the inoculated series was added to the controls, and they always were prepared and sown before preparing and planting the inoculated series in order to prevent accidental contamination.

The relative influences of the different temperatures and moistures were determined on the basis of the amounts of disease produced. The method used for determining the amount of disease was essentially the same as that used in the studies on the Helminthosporium disease of wheat (*12, p. 199–200*). However, owing to the fact that *Ophiobolus graminis* is a more vigorous parasite than *Helminthosporium sativum*, the wheat plants were more severly diseased in the experiments under discussion and it became necessary to classify the injuries in a different manner than was done in the previous studies referred to.

Disease manifestations were divided into three groups or types—(1) leaf and stem blight, (2) infection of tiller bases, and (3) root infection. In order that all data should represent the severity of infection as well as the number of plants infected, the various types of injury were given the numerical ratings shown in Table I. Instead of expressing the total amount of disease occurring at a given temperature as a single infection rating, the different types of injury were kept separate and the infection ratings were calculated for each independently. These calculations were made according to the method employed in the studies on the Helminthosporium disease previously referred to (*12, p. 200*).

TABLE I.—*Method of determining the numerical ratings for healthy and diseased wheat plants*

Type of injury	Class	Degree and type of injury	Numerical rating
Leaf and stem blight	1	None	0
	2	Leaves yellow	1
	3	Plant killed	2
Infection of tiller-bases	1	None	0
	2	Slight	1
	3	Moderate	2
	4	Abundant	3
Root infection	1	None	0
	2	Slight	1
	3	Moderate	2
	4	Abundant	3

RESULTS

HOST DEVELOPMENT

The best germination of Goldcoin wheat was obtained at soil temperatures ranging from 12° to 20° C. and at soil moistures ranging from 54.4 to 80 per cent of the moisture-holding capacity. As in the case of Harvest Queen and Marquis wheats (*12*), germination takes place first at the high temperatures and it is retarded at the low temperatures. During the periods of the experiments (20 to 31 days) the plants produced the greatest dry weight of the aboveground portions at temperatures ranging from 20° to 28° C. and at soil moistures ranging from 71.6 to 80 per cent. Root development seemed to be stimulated at soil temperatures ranging from 16° to 26°. All of the soil moistures employed in these experiments seemed to be about equally favorable for root development.

DEVELOPMENT OF THE PARASITE

One of the writers (Davis) has studied the influence of temperature on the growth of the parasite in pure culture, and the results are given in another paper (*1*). It was found that growth took place at temperatures ranging from 4° to 33° C. The optimum temperatures for growth varied somewhat for the strains of the parasite studied. However this variation was within the limits of 19° to 23°. The optimum for the New York strain was rather wide, growth seeming to proceed equally well anywhere between 19° and 24°, whereas the optimum temperature for the growth of the Oregon strain of the parasite seemed to be between 23° and 24°.

DISEASE DEVELOPMENT

In no case did Ophiobolus infection occur in any of the plants growing in the uninoculated controls (pl. 1, A). The results obtained in all of the inoculated experiments [4] are tabulated in Tables II, III, IV, V, VI, and VII, and the data are shown graphically in Figures 1 to 7, inclusive. Plate 1, B illustrates the results obtained on a representative one-tenth of the plants grown in the inoculated soil in Experiment 1B.

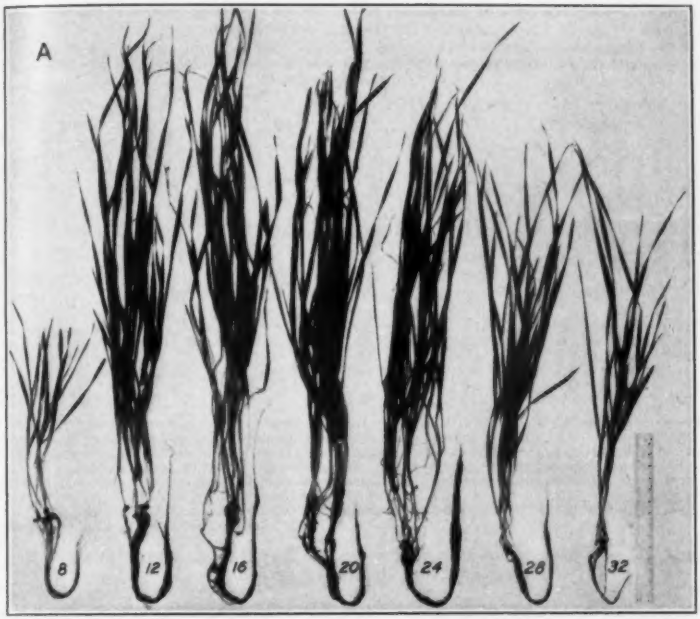
[4] In McKINNEY, H. H. TAKE-ALL AND FOOT-ROT INVESTIGATIONS. U. S. Dept. Agr., Bur. Plant Indus., Cereal Courier 14: 23-25. 1922. [Mimeographed] the writers reported a preliminary experiment in which the greatest amount of injury took place in soil held at 22° to 24° C. The population of this experiment was very small and the amount of parasite used was too great for accurate results, as shown by later experiments. Owing to these circumstances and the fact that data were based entirely on top injury, these results are not considered significant and are not included among the results of the subsequent experiments.

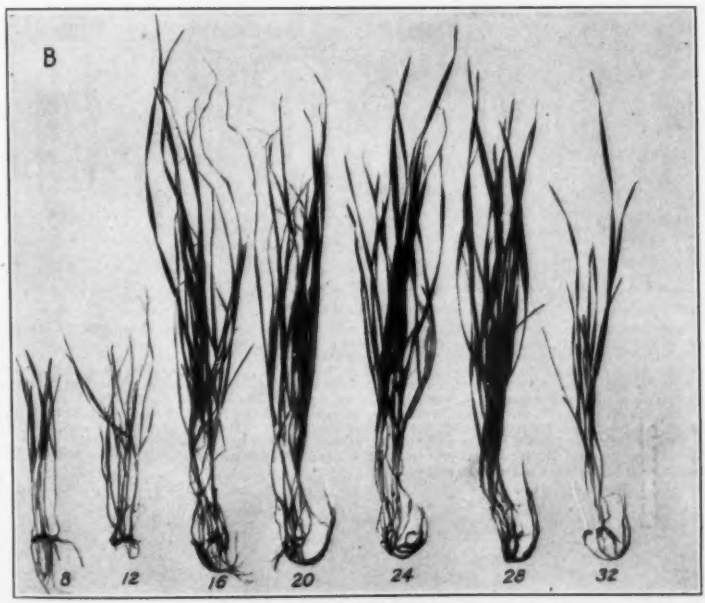
EXPLANATORY LEGEND FOR PLATE 1

Influence of soil temperature on the infection of Goldcoin wheat seedlings and young plants by *Ophiobolus graminis*

A.—A representative tenth of all the plants grown at the various soil temperatures in the uninoculated soil of experiment 1B. These plants were all free from infection, as was the case with all of the other uninoculated plants grown in all of the experiments

B.—A representative tenth of all the plants grown at the various soil temperatures in the inoculated soil of experiment 1B. Note the very severe infection in the plants grown at 12° C.; most of these plants died. Also note the severe root rotting on plants grown at soil temperatures below 24° C.





(For explanatory legend see p. 830)

TABLE II.—*Effects of soil temperature on the infection of young Goldcoin wheat plants with Ophiobolus graminis*

[These experiments were conducted independently of those in which soil temperature and soil moisture were varied simultaneously]

Experiment 1A [a]					Experiment 1B [b]					Summary [c]			
Average soil temperature, °C.	Number of plants	Leaf-blight rating	Tiller-base infection rating	Root-infection rating	Average soil temperature, °C.	Number of plants	Leaf-blight rating	Tiller-base infection rating	Root-infection rating	Average soil temperature, °C.	Leaf-blight rating	Tiller-base infection rating	Root-infection rating
8	26	0.0	0.6	33.0	8	31	1.6	13.9	25.2	8	0.8	7.2	29.1
12	26	8.3	32.0	45.5	12	29	30.4	42.5	45.9	12	19.3	37.2	45.7
16	26	3.2	28.2	43.5	16	23	17.3	42.0	42.0	16	10.2	35.1	42.7
20	32	.0	12.5	26.0	20	31	2.2	34.9	40.3	20	1.1	23.7	33.1
24	26	.0	4.5	7.7	24	28	.0	5.9	9.5	24	.0	5.2	8.6
28	26	.0	1.9	3.8	28	21	.0	7.1	3.1	28	.0	4.5	3.4
32	13	.0	3.8	.0	32	18	.0	2.8	4.6	32	.0	3.3	2.3

[a] Plants grown in soil having a moisture-holding capacity of 67 per cent and containing 56.1 per cent moisture; one 6-inch soil can used for each temperature; 35 seeds and 100 grams of inoculum containing the Oregon strain of the parasite used in each soil can; started Feb. 14, 1922; ended Mar. 10, 1922.

[b] Experiment carried on at the same time and in the same manner as experiment 1A, except that it was not ended until Mar. 17, 1922.

[c] Average amount of infection at each soil temperature in experiments 1A and 1B.

TABLE III.—*Effects of soil temperature on the infection of young Goldcoin wheat plants with Ophiobolus graminis*

[These results are from experiments which were a part of the soil-moisture experiments and comprise all of the data obtained in soil of relatively low-water content]

Experiment 2A [a]					Experiment 3A [b]					Experiment 4A [c]					Summary [d]			
Average soil temperature, °C.	Number of plants	Leaf-blight rating	Tiller-base infection rating	Root-infection rating	Average soil temperature, °C.	Number of plants	Leaf-blight rating	Tiller-base infection rating	Root-infection rating	Average soil temperature, °C.	Number of plants	Leaf-blight rating	Tiller-base infection rating	Root-infection rating	Average soil temperature, °C.	Leaf-blight rating	Tiller-base infection rating	Root-infection rating
8	34	2.9	10.7	75.9	8	39	0.0	35.9	56.0	8	48	0.0	14.5	38.8	8	0.9	20.3	56.9
12	26	42.3	79.4	84.0	12	46	.0	65.2	49.0	12	46	23.8	55.8	80.0	12	22.0	66.8	71.0
16	29	24.4	78.1	69.5	16	54	16.6	69.7	68.0	16	50	28.0	78.6	81.9	16	23.0	75.4	73.1
20	24	22.9	44.4	69.5	20	53	2.8	41.5	38.0	20	50	7.0	67.3	70.6	20	10.9	51.1	59.4
24	12	.0	2.8	5.5	24	53	2.8	40.8	47.0	24	51	9.8	38.5	62.7	24	4.2	27.3	38.4
28	20	5.0	.0	.0	28	45	.0	6.6	.0	28	43	.0	10.0	16.6	28	1.6	5.5	5.5

[a] Plants grown in soil having a moisture-holding capacity of 67 per cent and containing 33.8 per cent moisture; one 6-inch soil can used for each temperature; 35 seeds and 100 grams of inoculum containing the Oregon strain of the parasite used in each soil can; started June 3, 1922; ended July 1, 1922.

[b] Plants grown in soil having a moisture-holding capacity of 53 per cent and containing 33.2 per cent moisture; one 8-inch soil can used for each temperature; 60 seeds and 400 grams of inoculum containing the Oregon strain of the parasite used in each soil can; started Feb. 21, 1923; ended Mar. 14, 1923.

[c] Plants grown in soil having a moisture-holding capacity of 53 per cent and containing 33.2 per cent moisture; one 8-inch soil can used for each temperature; 60 seeds and 400 grams of inoculum containing the New York strain of the parasite used in each soil can; started Mar. 31, 1923; ended Apr. 20, 1923.

[d] Average amount of infection at each soil temperature in experiments 2A, 3A, and 4A, having soil moistures of 33.8, 33.2 and 33.2 per cent, respectively.

TABLE IV.—*Effects of soil temperature on the infection of young Goldcoin wheat plants with Ophiobolus graminis*

[These results are from experiments which were a part of the soil moisture experiments and comprise all of the data obtained in soil of medium water content]

Experiment 2B [a]					Experiment 3B [b]					Experiment 4B [c]					Summary [d]				
Average soil temperature, °C.	Number of plants	Leaf-blight rating	Tiller-base infection rating	Root-infection rating	Average soil temperature, °C.	Number of plants	Leaf-blight rating	Tiller-base infection rating	Root-infection rating	Average soil temperature, °C.	Number of plants	Leaf-blight rating	Tiller-base infection rating	Root-infection rating	Average soil temperature, °C.	Leaf-blight rating	Tiller-base infection rating	Root-infection rating
8	32	0.0	1.0	9.4	8	54	0.0	63.5	64.7	8	49	0.0	33.3	33.3	8	0.0	32.6	35.8
12	33	1.5	64.6	44.7	12	54	.0	69.1	70.0	12	49	70.4	94.5	98.5	12	23.9	76.0	71.0
16	31	11.2	18.2	10.7	16	51	36.2	97.4	76.4	16	57	72.8	94.7	99.5	16	40.0	70.1	62.2
20	27	16.6	3.7	12.3	20	42	30.9	92.0	79.0	20	51	71.5	94.8	93.1	20	39.7	63.5	61.4
24	20	.0	.0	.0	24	44	28.4	78.7	70.0	24	49	60.2	93.1	89.3	24	29.5	57.3	53.1
28	17	.0	1.9	.0	28	47	2.1	26.2	37.0	28	50	.0	64.0	66.0	28	.7	30.7	34.3
32	5	.0	.0	.0											32	.0	.0	.0

[a] This experiment was carried on at the same time and in the same manner as experiment 2A, except that the soil moisture was held near 54.4 per cent.

[b] This experiment was carried on at the same time and in the same manner as experiment 3A, except that the soil moisture was held near 55 per cent.

[c] This experiment was carried on at the same time and in the same manner as experiment 4A, except that the soil moisture was held near 56 per cent.

[d] Average amount of infection at each soil temperature in experiments 2B, 3B, and 4B having soil moistures of 54.4, 55, and 56 per cent, respectively.

TABLE V.—*Effects of soil temperature on the infection of young Goldcoin wheat plants with Ophiobolus graminis*

[These results are from experiments which were a part of the soil moisture experiments and comprise all of the data obtained in soil of relatively high water content]

Experiment 2C [a]					Experiment 3C [b]					Experiment 4C [c]					Summary [d]			
Average soil temperature, °C.	Number of plants	Leaf-blight rating	Tiller-base infection rating	Root-infection rating	Average soil temperature, °C.	Number of plants	Leaf-blight rating	Tiller-base infection rating	Root-infection rating	Average soil temperature, °C.	Number of plants	Leaf-blight rating	Tiller-base infection rating	Root-infection rating	Average soil temperature, °C.	Leaf-blight rating	Tiller-base infection rating	Root-infection rating
8	35	0.0	12.3	66.6	8	45	0.0	33.3	66.6	8	57	0.0	29.2	33.3	8	0.0	24.9	55.5
12	26	28.8	62.8	73.3	12	46	15.2	67.4	75.2	12	48	53.1	65.9	88.3	12	32.3	65.3	78.9
16	28	32.1	77.3	81.0	16	43	79.0	97.6	98.3	16	60	72.5	85.0	95.5	16	61.2	86.6	91.6
20	20	42.5	58.3	79.0	20	56	36.6	93.4	84.5	20	46	66.3	89.8	88.3	20	48.4	80.5	83.7
24	16	.0	18.7	43.7	24	37	67.5	94.5	96.4	24	48	76.0	88.2	96.8	24	47.8	67.1	78.9
28	19	.0	15.8	20.3	28	32	40.6	87.5	90.4	28	27	7.4	37.0	37.6	28	16.0	46.7	49.4
32	5	.0	6.6	20.0	32					32					32	.0	6.6	20.0

[a] This experiment was carried on at the same time and in the same manner as experiment 2A, except that the soil moisture was held near 71.6 per cent.

[b] This experiment was carried on at the same time and in the same manner as experiment 3A, except that the soil moisture was held near 80 per cent.

[c] This experiment was carried on at the same time and in the same manner as experiment 4A, except that the soil moisture was held near 80 per cent.

[d] Average amount of infection at each soil temperature in experiments 2C, 3C, and 4C having soil moistures of 71.6, 80, and 80 per cent, respectively.

TABLE VI.—*Effects of variations in soil and air temperature on the infection of young Goldcoin wheat plants with Ophiobolus graminis*

[The plants were grown in large chambers in which both temperature and humidity were controlled within relatively close limits. The soil was inoculated with the New York strain of the parasite, and 15 disinfected seeds were planted in each of six containers for each temperature chamber. Experiment started March 31, 1923, and ended April 27, 1923]

Temperature range, ° C.	Number of plants	Tiller-base infection rating	Root-infection rating
9.5 to 13.2	70	76.3	97.9
15.8 to 17.0	68	85.1	96.4
26.5 to 27.5	59	21.4	36.1

TABLE VII.—*Summary of data given in Tables III, IV, and V, arranged to show the influence of soil moisture on the infection of young Goldcoin wheat plants with Ophiobolus graminis*

Leaf blight				Tiller-base infection				Root infection			
Average soil temperature, ° C.	Low soil moisture	Medium soil moisture	High soil moisture	Average soil temperature, ° C.	Low soil moisture	Medium soil moisture	High soil moisture	Average soil temperature, ° C.	Low soil moisture	Medium soil moisture	High soil moisture
8	0.9	0.0	0.0	8	20.3	32.6	24.9	8	56.9	35.8	55.5
12	22.9	23.9	32.3	12	66.8	76.0	65.3	12	71.0	71.0	78.9
16	23.0	40.0	61.2	16	75.4	70.1	86.6	16	73.1	62.2	91.6
20	10.9	39.7	48.4	20	51.1	63.5	80.5	20	59.4	61.4	83.7
24	4.2	29.5	47.8	24	27.3	57.3	67.1	24	38.4	53.1	78.9
28	1.6	.7	16.0	28	5.5	30.7	46.7	28	5.5	34.3	49.4
32	-------	0	.0	32	-------	.0	6.6	32	-------	.0	20.0

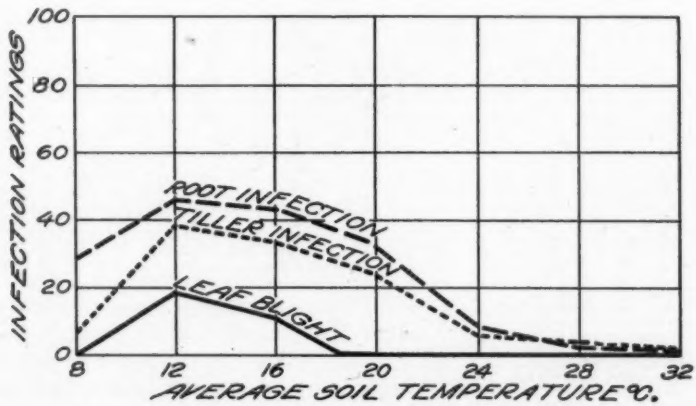


FIG. 1.—Curves showing summaries of infection ratings on the roots, tiller bases, and aboveground parts of Goldcoin wheat plants inoculated with *Ophiobolus graminis*, when grown in soil containing 56.1 per cent of moisture, and held at different temperatures, as shown in Table II

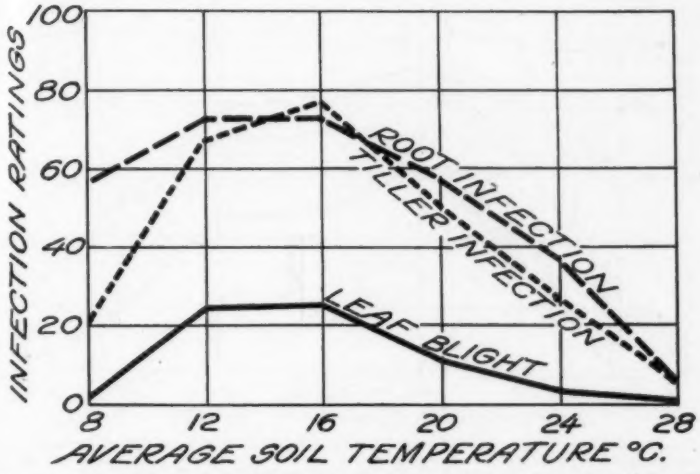


FIG. 2.—Curves showing summaries of infection ratings on the roots, tiller bases, and aboveground parts of Goldcoin wheat plants inoculated with *Ophiobolus graminis*, when grown in soil containing from 33.2 to 33.8 per cent of moisture, and held at different temperatures, as shown in Table III

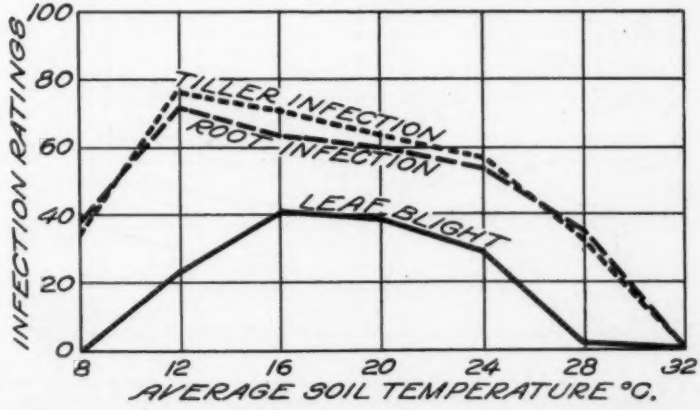


FIG. 3.—Curves showing summaries of infection ratings on the roots, tiller bases, and aboveground parts of Goldcoin wheat plants inoculated with *Ophiobolus graminis*, when grown in soil containing from 54.4 to 56 per cent of moisture, and held at different temperatures, as shown in Table IV

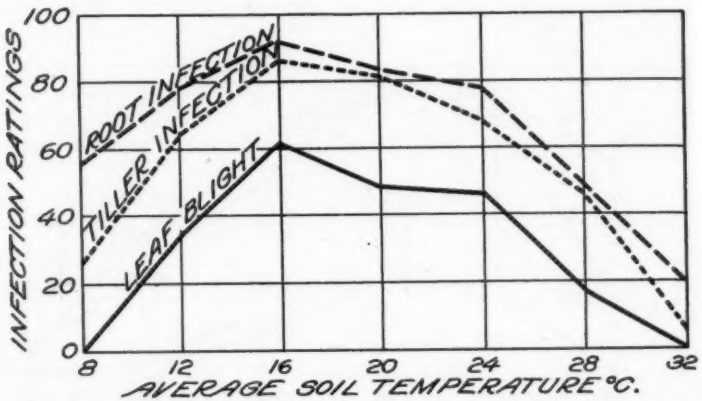


FIG. 4.—Curves showing summaries of infection ratings on the roots, tiller bases, and aboveground parts of Goldcoin wheat plants inoculated with *Ophiobolus graminis*, when grown in soil containing from 71.6 to 80 per cent of moisture, and held at different temperatures, as shown in Table V

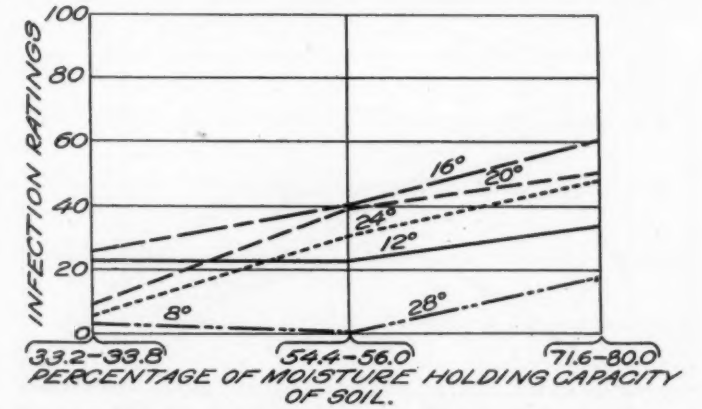


FIG. 5.—Curves showing summaries of infection ratings on the aboveground parts of Goldcoin wheat plants inoculated with *Ophiobolus graminis*, when grown at different soil moistures when soil temperature was varied simultaneously. Tabular results are given in Table VII

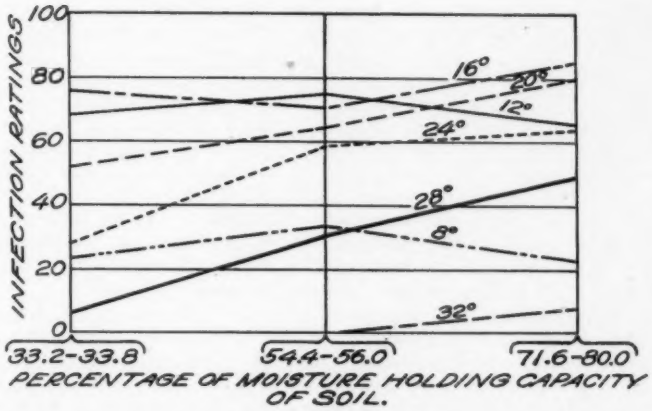


FIG. 6.—Curves showing summaries of infection ratings on the tiller bases of Goldcoin wheat plants inoculated with *Ophiobolus graminis*, when grown at different soil moistures when soil temperature was varied simultaneously. Tabular results are given in Table VII

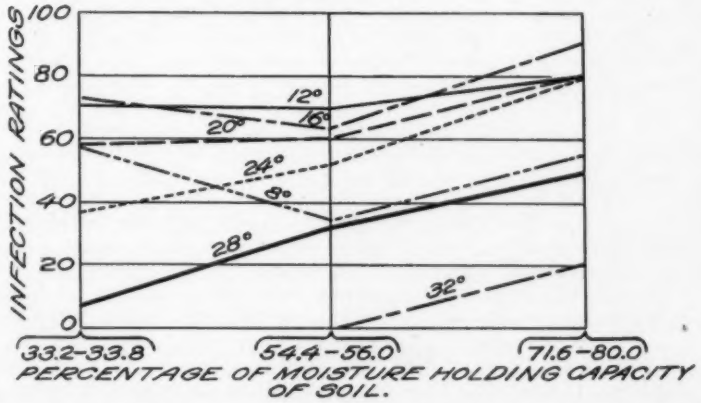


FIG. 7.—Curves showing summaries of infection ratings on the roots of Goldcoin wheat plants inoculated with *Ophiobolus graminis*, when grown at different soil moistures when soil temperature was varied simultaneously. Tabular results are given in Table VII

From the data presented, it is evident that *Ophiobolus graminis* is a vigorous root and tiller-base parasite, and also that infection is greatly influenced by soil temperature and soil moisture. Although the temperature and moisture optima shifted slightly in the several experiments, it is evident that infection and injury are favored by moderately low temperatures (12° to 16° C.) and by fairly high soil moistures (70 to 80 per cent). In these studies it was found that *O. graminis* causes practically no injury to the very young seedlings just before emergence. The germination of seeds planted in the inoculated soils was not consistently lower than that of seeds sown in the uninoculated soil; in fact it was not uncommon to find that the germination of seed in the control soil was actually lower than that of seeds sown in the inoculated soil. It was found in these studies that infection seldom took place until the seedlings were well advanced. This was found to be the case even in the experiments in which the greatest quantities of inoculum were used. When small quantities of inoculum were used the plants showed little infection until they were well advanced beyond the seedling stage.

An examination of the summary curves in Figures 2, 3, and 4 shows that the optimum temperature for tiller-base and root injury was 12° C. in soil containing the medium amount of water, whereas 16° C. was the optimum temperature for these injuries in soils containing low and high soil moistures. Whether this shift represents an actual relationship between the joint influences of temperature and moisture or merely experimental variation is a question. The data given for the individual experiments show that the temperature optima shifted from time to time, regardless of the moisture content of the soil. This indicates that some other factors which were not so well controlled as soil temperature and soil moisture may have a decided influence on the occurrence of the disease and that the temperature optimum probably extends over a range of at least 4° to 6°. The quantity of Ophiobolus inoculum placed in the soil has a marked influence on the behavior of the take-all disease, as was also found in the studies on *Actinomyces scabies* (*8*) and *Helminthosporium sativum* (*12*). However, it is much more difficult to standardize the amount of inoculum of *Ophiobolus graminis* than is the case with the inoculum of the other parasites mentioned, and as the strains of *O. graminis* employed in these studies differed in their virulence it was frequently found that the quantity of inoculum used was in excess of that which would give the sharpest indications of the temperature and moisture optima.

DISCUSSION

Although these studies have only opened up the general subject of environmental influence on the development of the take-all disease, it is believed that the data presented represent the general influences of temperature and moisture on the infection of wheat seedlings and young wheat plants by *Ophiobolus graminis*. While it is difficult or impossible to correlate many of the field observations which have been recorded on this subject, it now appears from the experimental data herein presented that some of the seemingly contradictory reports may be in accord with the facts. As pointed out earlier, the quantity of infectious material in the soil influences the amount and severity of take-all, and this relationship doubtless is the basis for many discrepancies in field observations.

In these experiments of the writers it was found that all infected plants did not show signs of the disease on the aboveground parts. In many cases plants which appeared healthy before removal from the soil were in reality almost devoid of a root system. This condition has been noted also by one of the writers (McKinney) in several commercial wheat fields which were affected by take-all. Although data presented here show that relatively high soil moistures favor infection and injury, it is evident that these soil moistures also were favorable for the development of the host. At these soil moistures many plants sent out new roots in an attempt to replace those which had become rotted. In the present studies the writers did not subject any particular lot of plants to various temperature and moisture changes in order to determine the exact influence of hot, dry conditions following a period of cold, moist conditions or vice versa. However, on a basis of our knowledge of plant responses it is only reasonable to assume that even though cool, moist conditions do favor infection and injury, a sudden reduction in water supply and increased transpiration would produce marked leaf yellowing and killing among those apparently healthy plants which in reality have badly rotted culms and roots. One of the writers (McKinney) has noted an increase in the amount of leaf yellowing in infested fields after the beginning of a hot, dry period. Under such conditions, it might be assumed that dry, hot weather actually favors infection, but it appears that such weather only brings out the expression on the aboveground parts of injuries below ground which are favored by the opposite set of conditions. It would seem that the nonappearance, in the fall and extremely early spring, of take-all on winter wheat on infested land is probably accounted for on a basis of cool, moist conditions which favor the growth of the wheat plants although already infected. However, when the warm spring days arrive the infected plants turn yellow and finally die.

Although the optimum temperature for the development of the parasite is more limited than that for the host, it is of interest to note that the two optima appear to be very close together. On the other hand, the optimum temperature range for the disease is considerably lower than that for either the host or parasite. This relationship is exactly the reverse of that existing with the Helminthosporium disease of wheat, the latter disease being favored by soil temperatures which are above those favorable for the host and parasite. In a paper dealing with this latter disease, McKinney (*12*) suggested that the high temperature optimum for disease occurrence might be accounted for on the basis of the weakening of the host at the high, unfavorable temperatures. However, it is not possible to explain the results with *Ophiobolus graminis* on such a basis. At 12° and 16° C. the wheat plant is thrifty and robust, yet it is attacked severely by this parasite. *Ophiobolus graminis* produces severe injury at unusually low temperatures in comparison with the other wheat parasites such as *Gibberella saubinetii*, studied by Dickson (*2*), and *Helminthosporium sativum*, studied by the senior writer (*12*). It seems that the explanation of this will require the study of factors relating to both the host and the fungus and which are far more basic than the phenomena of growth rate and vigor as expressed by the ordinary methods of weight and measurement. It is believed that this also applies to the explanation of the behavior

of the Helminthosporium disease. This is supported by the results obtained by Dickson, Eckerson, and Link (*3*) with two hosts (corn and wheat) which possess different disease-temperature optima when attacked by *G. saubinetii*. It will be especially important to investigate these problems further, not only by the use of additional hosts and one parasite but by employing one host and several parasites which possess different disease-temperature optima. Unquestionably *H. sativum*, *O. graminis*, and the wheat plant offer opportunities in this direction.

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BACTERIAL SPOT OF COWPEA AND LIMA BEAN[1]

By MAX W. GARDNER, *Associate in Botany*, and JAMES B. KENDRICK, *Assistant in Botany, Purdue University Agricultural Experiment Station*[2]

INTRODUCTION

The bacterial spot disease of cowpeas was first noted in southern Indiana in 1919, but its bacterial nature was not determined until 1921, when it occurred in an experimental plot of cowpeas at La Fayette, Ind. It is a typical spot disease of the leaves, stems, and pods, distinctly different from the other bacterial diseases of cowpeas reported in the literature. The organism which causes this disease also causes a very similar and widespread spot disease of lima beans which has been described recently by Tisdale and Williamson (*23*, *24*).[3] The work herein reported deals mainly with the symptoms of the disease as it occurs on cowpeas, the isolation, characteristics, overwintering, pathogenicity, and dissemination of the causative bacteria, and the identity of the latter with the species causing the lima-bean disease.

HISTORY AND OCCURRENCE

A search of the literature has not revealed any previous description of this disease of cowpeas, although the symptoms of the spot disease of lima beans and cowpeas described by Smith (*18*, *p. 15*) in 1905, and attributed to *Phyllosticta phaseolina* Sacc., closely resemble those of the disease under consideration. A number of cases of bacterial infection of cowpeas have been recorded. Smith and McCulloch (*19*), in 1919, reported a wilt of cowpeas produced by inoculation with *Bacterium solanacearum* E. F. S. Smith (*20*, *p. 280*) mentions a bacterial spot of cowpeas, but does not describe the disease. Rapd (*15*, *p. 3*) in 1920 and more recently Burkholder (*3*, *p. 7*) have reported infection of cowpeas with *Bacterium phaseoli* E. F. S., an organism which, however, differs radically in culture from the organism causing the spot disease. Wolf and Foster (*25*, *p. 452*) have isolated the tobacco wildfire organism (*Bact. tabacum* Wolf and Foster) from small, yellowish leaf spots on cowpeas grown near infected tobacco, but attribute the cowpea lesions to infection about leafhopper wounds. Osmun (*14*) has isolated the wildfire organism from lima beans grown adjacent to tobacco, and Johnson, Slagg, and Murwin (*10*, *p. 177*) successfully inoculated cowpeas with the organism. However, this organism differs culturally from the one causing the cowpea spot disease, and the writers' attempts to inoculate tobacco have failed. The history of bacterial spot as it occurs on lima bean has been given by Tisdale and Williamson (*24*). Chupp has reported to the Federal Plant Disease Survey the presence since 1918 of the disease on lima beans in New York.

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[1] Received for publication Nov. 11, 1924; issued January. Contribution from the Department of Botany, Purdue University Agricultural Experiment Station, La Fayette, Ind.

[2] The writers wish to acknowledge their indebtedness to Prof. H. S. Jackson for his advice and criticism.

[3] Reference is made by number (italic) to "Literature cited," p. 862.

The disease on cowpeas was first found at Vallonia, Ind., in August, 1919, and was next found at Decker, in southern Indiana, in August, 1920. In August, 1921, it occurred in an experimental plot of Whippoorwill cowpeas at La Fayette, Ind., and has occurred in the writers' plots in 1922, 1923, and 1924. The disease appears to be very widespread, since it has been found in seed from South Carolina, Virginia, and Washington, D. C., and was noted in the field in 1922 near Decker, Ind., Seaford, Del., and Louisville, Ky. Seiyo Ito, who examined the writers' plots at La Fayette, said that the same disease also occurs in Japan.

In 1923 the disease was found in Knox County, Ind., and in Kansas by R. P. White, and in Florida by W. B. Tisdale, who also reported it as a serious trouble in 1924. In 1924 the disease was serious in the field crop (variety, New Era) in Jackson County, Ind., and was more severe in the writers' plots at La Fayette than in the preceding three years. This disease as it occurs on lima beans was noted commonly in gardens in Indiana in 1919 and 1920, and it occurred to a considerable extent in 1923 and 1924.

HOSTS

Greenhouse inoculations and field studies have shown that the following are hosts of this parasite: Cowpea (*Vigna sinensis* [L.] Endl.); catjang (*Vigna catjang* Walp.); hyacinth bean (*Dolichos lablab* L.); Florida velvet bean (*Stizolobium deeringianum* Bort); adsuki bean (*Phaseolus angularis* Wight); lima bean (*Phaseolus limensis* Macf.), as represented by the Large White Pole, Giant Podded Pole, and King of the Garden varieties; bush lima bean (*Phaseolus limensis* Macf. var. *limenanus* Bailey), as represented by the Burpee's Bush and Fordhook varieties; and the dwarf sieva bean (*Phaseolus lunatus* L. var. *lunonanus* Bailey), as represented by the Henderson's Bush variety. Tisdale and Williamson (*24*) had found the varieties Burpee's Bush, Fordhook, King of the Garden, Dreer's Bush, and Henderson's Bush to be susceptible. Natural infection with what is in all probability the same organism was found rather abundantly on the leaves of the common native weed, tick trefoil (*Desmodium canescens* [L.] DC.), in a fallow field near La Fayette, Ind., on August 23, 1924, and has also been found on asparagus bean (*Vigna sesquipedalis* Wight) in field plots.

All of the following 23 varieties of cowpeas tested have proved to be susceptible: Whippoorwill, Brabham, Early Red, Early Black, Early Buff, Taylor, Black, Red Ripper, Iron, Conch, Groit, New Era, Clay, Wonderful, California Blackeye, Early Ramshorn Blackeye, Cream Chowder, Gallavant, Large Blackeye, Arlington, Columbia, Progressive White, and Victor.

SYMPTOMS

On the cowpea leaves, the spots as usually seen in the field are irregularly circular or lobed rather than angular, and are 1 to 4 mm. broad, sometimes larger. Very young spots are small, circular, sunken dots, first water soaked or greasy, later claret-brown in color (*16*) (pl. 1, A). The larger lesions are characterized by a buff center surrounded by a conspicuous maroon or claret-brown margin about 1 mm. in width (pl. 1, B; pl. 2, A). Leaf lesions frequently become

A.—Lower surface of cowpea leaflet, showing small sunken lesions; and, on lower left side, a large lesion extending along a vein. ×2
B.—Upper surface of cowpea leaflet, showing lesions, older than those in A, with light centers and reddish-brown margins. In some the centers have fallen out. The large lesion near left-hand edge has an extension along a vein. ×2

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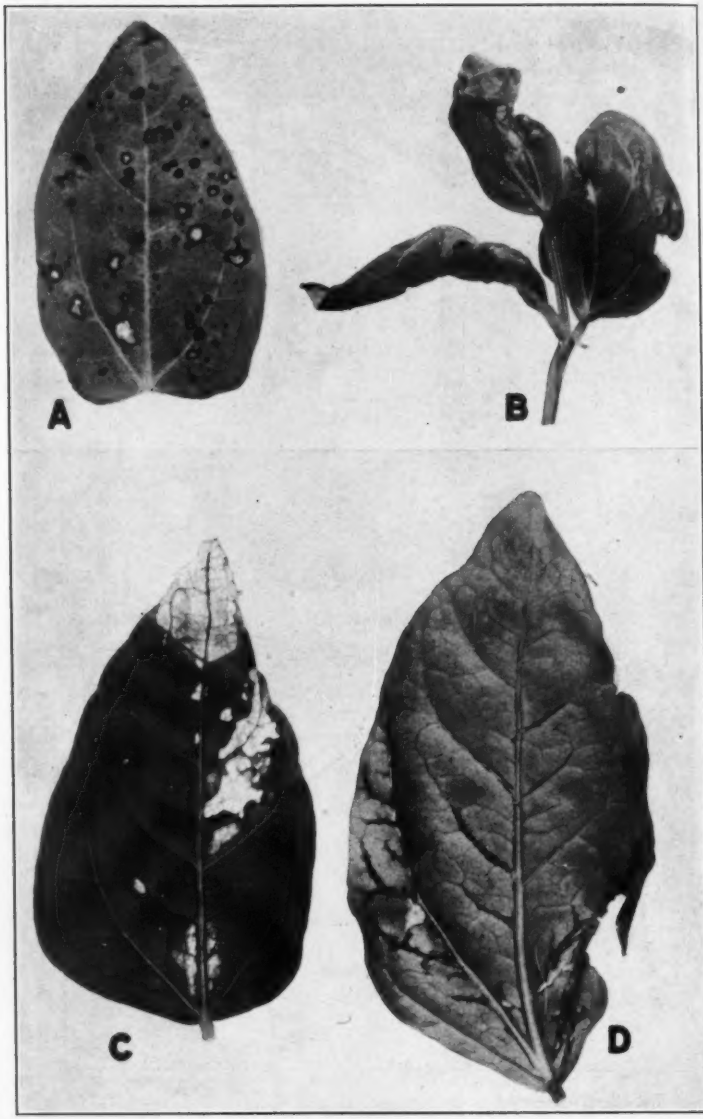


A.—Catjang leaflet, showing bacterial spot lesions
B.—Cowpea leaf. Infected early along the veins. Note distortion
C.—Cowpea leaflet. Infected early. Note bleached areas
D —Lower side of cowpea leaflet, showing invasion along the vein

rather large, bleached, dried areas surrounded by a claret-brown border (pl. 3, B). In old lesions the central tissue dries and may crack or drop out. Frequently the central tissue is invaded by fungi.

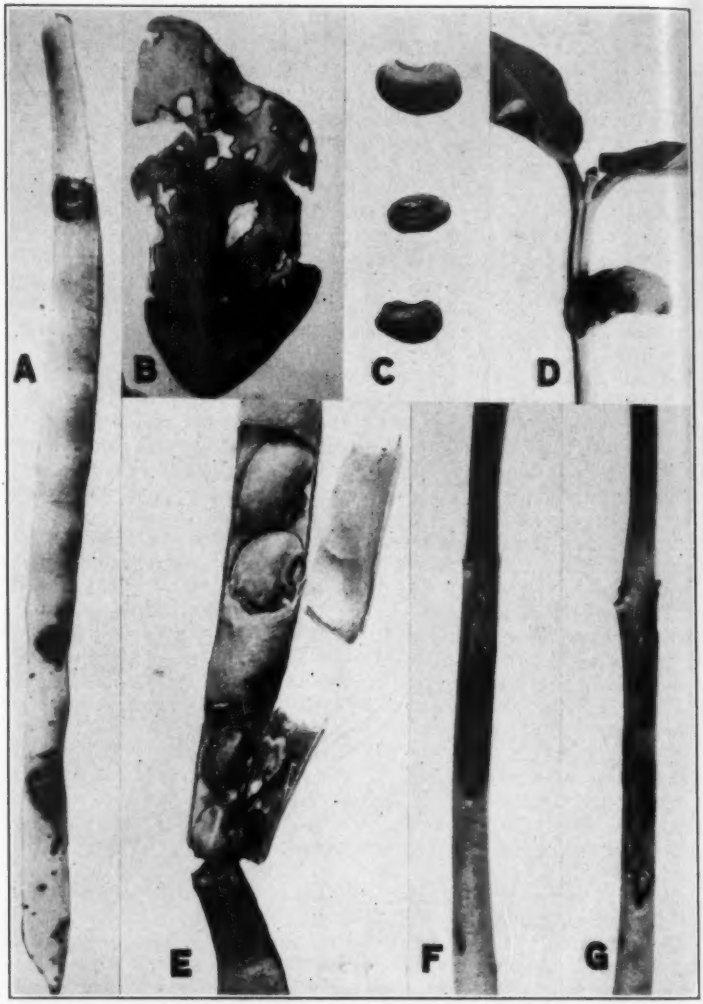
The lesions are not in any way delimited by the veins; on the contrary they frequently extend markedly along the veins (pl. 2, D; pl. 4, D and E). Linear leaf lesions have been noted extending along a vein a distance of 4 to 6 cm. If young leaf lesions are mounted in a drop of water on a slide and cut across with a scalpel, the bacteria may readily be seen under the microscope, oozing out in cloudy masses from the cut edge, especially from the region of the veins.

Infection occurs very readily on young growing leaves, and considerable distortion, curling, tearing, and puncturing of the leaves may be caused by the growth stresses (pl. 2, B; pl. 3, B). Lesions of early inception may thus become maroon-bordered slits and tears in the leaf lamina or along its margin, and vein lesions may cause curvature and crinkling. Lesions on a vein may cause a yellowing or bleaching (pl. 2, C) of distal portions of the leaf.

Lesions on the young leaves of cowpea seedlings under greenhouse conditions first become visible as small sunken pits or craters in the epidermis (pl. 5, C), which are translucent in transmitted light but soon become greasy or water-soaked and later claret-brown in color. At first such lesions are visible only on the lower side of the leaf and as a rule remain more extensive and more conspicuous there than on the upper side of the leaf. Older lesions assume the buff center and the claret-brown border and are surrounded by a narrow light-green halo. Also very small claret-brown lesions occur on the stipules (pl. 6, D). The symptoms on lima-bean leaves, described by Tisdale and Williamson (*24*), are essentially similar to those on cowpea leaves, as are also the lesions on catjang leaves (pl. 2, A). On the leaves of artificially infected velvet-bean plants the lesions were dark brown to black, with some tendency to be delimited by the veins (pl. 6, F). Natural infection of velvet-bean leaves produced lesions more closely resembling those on cowpea. These were irregularly circular, with a tan center and a dark-brown marginal line, and were surrounded by a light-green or yellowish halo in the living tissue. On tick trefoil the lesions showed a tan to light-brown center, with a narrow, reddish-brown marginal line, and were surrounded by a light-green or yellowish halo in the living tissue (pl. 5, B).

On the cowpea stems and petioles the lesions are more or less oval, 1 to 5 mm. long, and Victoria lake in color (pl. 6, D and E). The center is usually sunken, and there may be water-soaked tissue above and below the lesion. Frequently the lesions are much longer, especially on the petioles, and large sunken lesions are formed on the epicotyls and hypocotyls of seedlings (pl. 3, F and G). Similar stem and petiole infection also occurs on lima beans. Catjang leaves have been noticed to break off at petiole lesions.

The lesions on cowpea and catjang pods are irregularly circular and 1 to 8 mm. in diameter, and morocco red, claret brown, maroon, or Victoria lake in color (pl. 3, A). The larger lesions often have a sunken center and a water-soaked outer border. Infection of young pods results in a marked constriction of the pod at the point of infection, and usually in an abnormal bending of the pod at that



A.—Full-grown cowpea pod, showing lesions which have not caused conspicuous constrictions

B.—Cowpea leaflet, showing large lesions with tan centers and reddish brown borders. Such lesions result from early infection and cause malformation and shattering of leaves

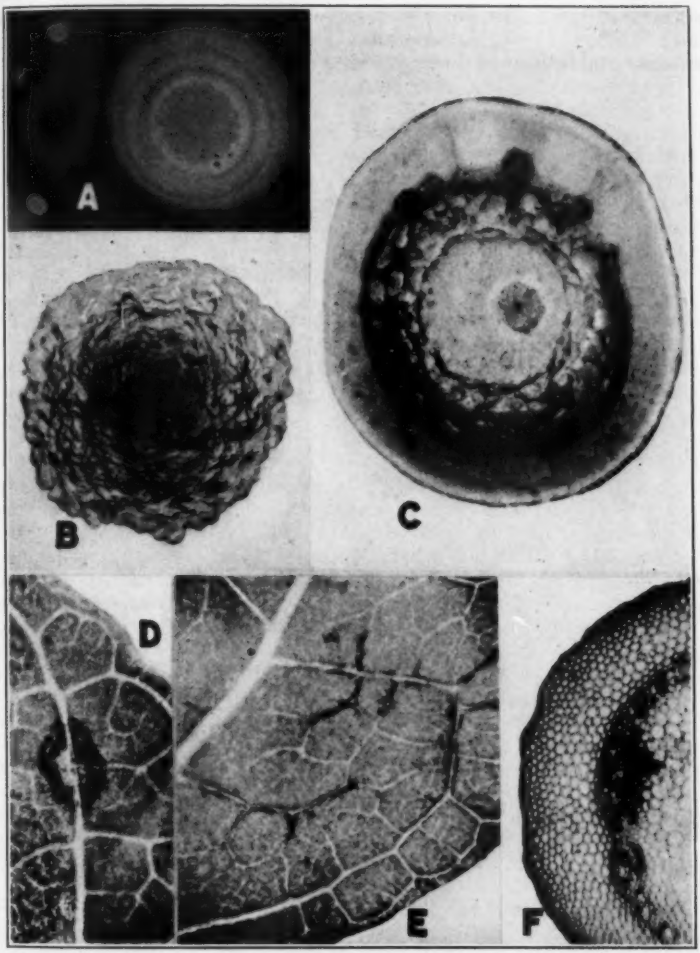
C.—Normal cowpea seed above. Two infected, shriveled, discolored seeds below, for comparison. × 2

D.—Seedling from an infected seed, showing the cotyledon lesion. × 2

E.—Portion of cowpea pod, showing the large normal seeds in the healthy part of the pod and the small, dark, infected seeds under a bacterial-spot lesion. × 2

F.—Portion of seedling stem, showing a large lesion extending down the hypocotyl from the point of attachment of the cotyledon. Such a lesion is the result of invasion from a cotyledon lesion such as is shown in D. × 2

G.—Hypocotyl of seedling enlarged (× 2) to show sunken lesions



A.—Agar-plate surface colony, with smaller submerged colonies at left. × 5
B.—Surface colony on a very dry agar, showing lobed margin and surface sculpturing. × 20
C.—Surface colony such as that shown in A, showing internal pattern. × 17
D.—Cowpea leaf lesion, showing that the veins do not limit the spread of the invasion. × 9
E.—Infection extending along the veinlets in a cowpea leaf. × 9
F.—Cross section of hypocotyl of cowpea, showing bacterial invasion of xylem elements as a result of infection proceeding from a cotyledon lesion. × 34

point (pl. 6, A, B, and C). Sometimes the entire distal portion of the pod fails to enlarge. Large lesions frequently penetrate through the ovary wall to the seed, causing a stunting, shriveling, and dark discoloration of the latter (pl. 3, C and E). However, attempts to separate upon this basis the infected from the healthy in commercial seed, have been unsuccessful. Tisdale and Williamson (*24*) found similar pod lesions and resultant seed infection in the lima bean.

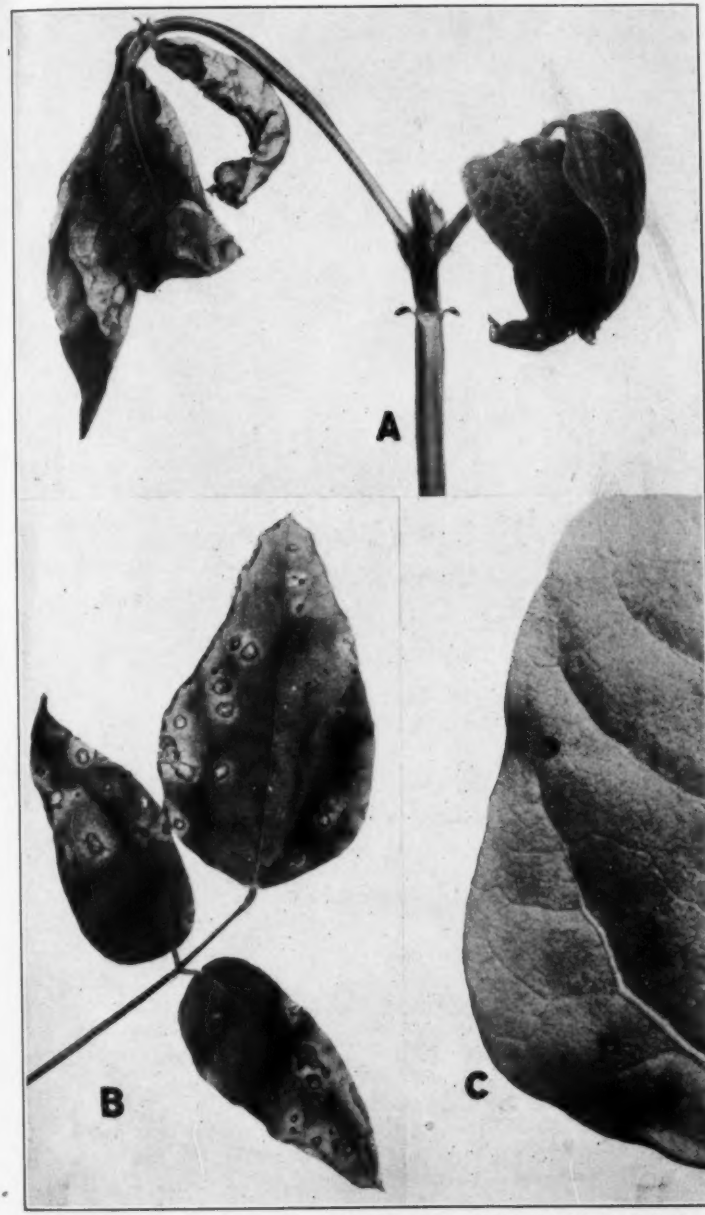
In cowpea seedlings from infected seed the cotyledons bear large lesions which frequently cause a water-soaking or dark discoloration and a shriveling or constriction of a considerable portion of the cotyledon (pl. 3, D). Such lesions are frequently accompanied by a transverse crack or fissure, and may kill the cotyledon prematurely. Oval, maroon lesions also occur on the hypocotyls and epicotyls of such seedlings (pl. 3, F and G).

From the infected cotyledons or from epicotyl lesions, infection of the vascular bundles occurs (pl. 4, F), and the invasion frequently extends up along one or more bundles through the petiole and out into the veins of the first leaf. This type of infection is visible as an internal reddish-brown streak within the vascular bundles, and causes a yellowing or wilting and blighting of all or portions of one or both of the first leaves. The affected portions of such leaves may show a darkened network of veins (pl. 2, C), and such infection has been observed to cause a rather extensive, shiny, brownish discoloration of the lower surface of the leaf along the veins. In some cases a preliminary yellowing of the veinlets and a stunting of the corresponding half of each leaf was observed. In extreme cases the entire seedling may be stunted or may wilt and die. Similar wilting and blighting effects have been noted on the first compound leaves of fieldgrown seedlings (pl. 5, A), and also on older plants as a consequence of early infection of the petiole which had resulted in extensive internal invasion of the vascular tissues. Under such conditions, irregular bleached areas of considerable extent frequently occurred on the leaf blades (pl. 2, C), accompanied sometimes by an extensive reddish discoloration of the lower epidermis.

The tendency to invade the veins has been noticed in the case of lesions on or near the veins of young leaves, in which case a brown streak may be traced out through or along the vein and into its smaller branches (pl. 1, A, B; pl. 2, D). Such infection may cause a yellowing or wilting of portions of the lamina supplied by the infected vein. Thus, although this is typically a spot disease, there are distinct evidences of localized vascular infection, particularly in the seedlings, which results in symptoms of a systemic nature.

In the Early Buff variety of cowpea this disease should not be confused with another spot disease with which a species of Cladosporium is associated. The latter trouble is characterized by smaller purple to black spots on the pods, irregular in outline, and by oval, sunken, purplish spots on the young stems and peduncles. These lesions may have a tan center on which appears a greenish, velvety, fungous growth. Pod infection occurs very early and often causes great distortion of the pods.

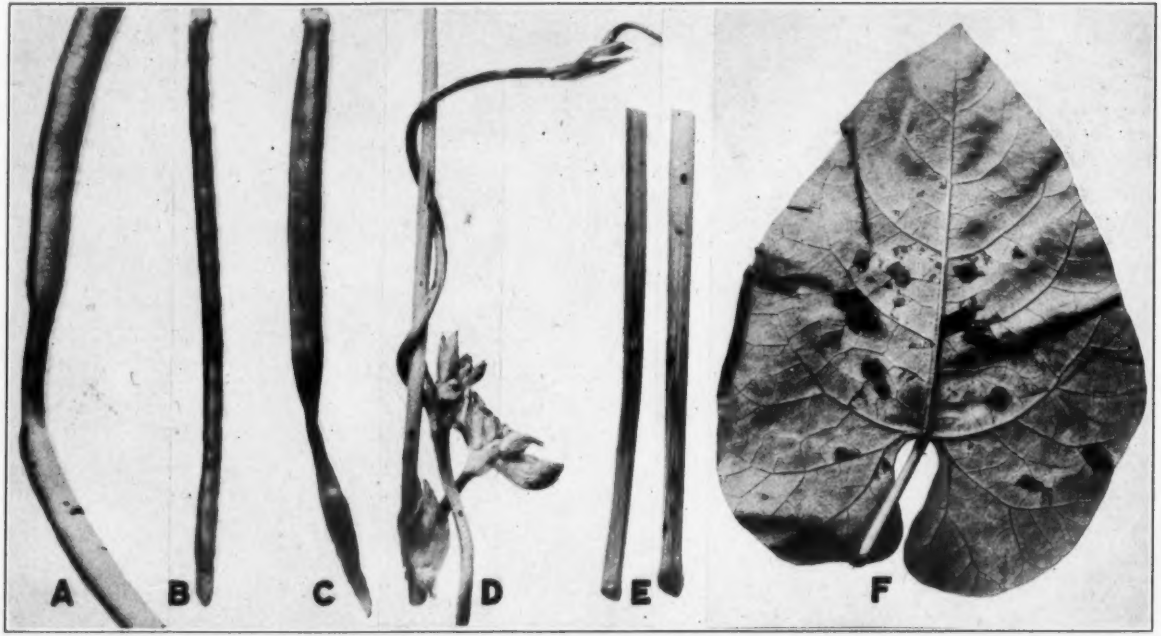
Early stages of the leaf spot caused by *Amerosporium oeconomicum* E. and T., as observed on the New Era variety, somewhat resemble bacterial spot, but the white center, concentric rings, and pycnidia of older lesions serve as distinguishing characters. The



A.—Wilting of leaves of field-grown cowpea seedling as a result of vascular infection in the stem and petiole

B.—Lesions on leaf of tick trefoil resulting from natural infection. The dry centers are tan or light brown, surrounded by a narrow dark-brown margin and a light-green or yellow halo

C.—Craterlike lesions on upper side of a first leaf of a cowpea seedling as a result of seed-borne infection ×3



A.—Portion of immature cowpea pod, showing a constriction due to two lesions resulting from early infection
B.—Young cowpea pod, showing stage at which growth is arrested by infection which results in such constrictions as are seen in A and C
C.—Young cowpea pod failing to enlarge at the infected point
D.—Lesions on stems and bracts of cowpea
E.—Lesions on petioles of cowpea
F.—Lesions on velvet bean leaflet resulting from atomizer inoculation

early stages of the leaf lesions caused by *Cercospora cruenta* Sacc. are more diffuse and less clearly defined. The older lesions are larger than the bacterial lesions, with a darker center and less conspicuous border than the latter, and show some tendency, at least in the Blackeye variety, to be delimited by the larger veins. The abundant sporulation of the fungus is of course a reliable differential characteristic.

ECONOMIC IMPORTANCE

The bacterial spot disease may cause very severe foliage injury to cowpea seedlings and young plants, especially in wet seasons, and not only kills leaves but may even cause the death of many young plants. Tisdale has reported through the Federal plant disease survey that this disease caused a serious defoliation of cowpeas in Florida. In the cowpea crop grown for seed the pod lesions of this disease may cause considerable loss. In the case of lesions constricting and stunting the pod, the number of seeds is reduced, and seeds borne under lesions are stunted or shriveled, are impaired in germinability, and produce diseased and weakened seedlings. Leaf and stem infection on older plants is, as a rule, less destructive. The disease seems to be very destructive to lima beans, especially in its leaf attack (*24*).

CAUSAL ORGANISM

ISOLATION

The bacterial nature of the disease on cowpeas was discovered in the late summer of 1921. On August 26 the surface of a spotted pod was sterilized in a 1:1,000 solution of mercuric chloride and a lesion was cut out with a flamed scalpel and macerated in a drop of sterile water on a flamed slide. This drop of water was plated out in potato-dextrose agar by the loop-dilution method, and after three days at room temperature the plates were evenly seeded with similar grayish-white bacterial colonies. The surface was sliced off from another pod lesion and a portion of the underlying brown tissue of the ovary wall was similarly tested. The plates were evenly seeded with colonies similar to those from the other lesion.

On the same date two leaf lesions were cut out with flamed scissors, immersed first in alcohol to wet the surfaces and then in a 1:1,000 solution of mercuric chloride for a few minutes, rinsed in sterile water, and macerated in drops of sterile water on flamed slides. The loop-dilution plates from both lesions were evenly seeded with grayish-white colonies apparently identical with those in the plates from the pod lesions. Furthermore, 18 leaf lesions were similarly cut out, sterilized, rinsed, and planted in poured plates of potato-dextrose agar. Grayish-white bacterial growth occurred around nine of these. Poured plate isolations made on August 30 from leaf, pod, and stem lesions yielded a similar organism. Numerous transfers were made from typical plate colonies to dextrose-potato agar slants and all appeared to be similar. Successful atomizer inoculations were made in the field and greenhouse, and the organism was reisolated from the lesions produced.

Successful isolations were made later from seedling cowpeas grown in sterile soil from diseased commercial seed. The organism was obtained from lesions on cotyledons, first leaves, epicotyl, and hypocotyl, and from vascular infection in the epicotyl. In 1923 the

organism was isolated also from lima beans grown in home gardens and from those exposed to infection in the experimental plots at the Purdue Experiment Station.

For the subsequent detailed study three strains were used, one an isolation from a cowpea-leaf lesion made August 26, 1921, and two were reisolations made in September, 1921, from cowpea-leaf lesions produced by field inoculations. These strains were tested for purity by poured plates and for pathogenicity by inoculations.

MORPHOLOGY

The organism is a rather small rod with rounded ends, and occurs singly or in pairs. The rods stain more readily in gentian violet than in Ziehl's carbol fuchsin or rose bengal. In agar cultures 24 hours old the cells varied in width from 0.44 to 0.66 μ, and in length from 1.10 to 2.34 μ, with an average of about 0.54 by 1.47 μ.

To obtain actively motile cells for the flagella stain, a piece was cut out of a 48-hour-old culture on potato-dextrose agar at the base of the slant and dropped into a sterile water blank. After four hours an examination of a hanging drop showed an abundance of motile cells and indicated that the organisms had diffused throughout the liquid. Smears were made from this water culture, and the flagella were stained by Van Ermengem's method. One to five flagella were found at one or both poles. Considerable variation was found, but more frequently there were three or four flagella at one pole and fewer at the other. Five flagella were noted at each pole in some instances. In another culture the predominating condition was that of one or two flagella at only one end of the rod. A few flagella were measured and averaged 6.5 μ in length.

Endospores, capsules, and involution forms have not been noted. In water suspensions 30 hours old the cells become swollen and vacuolated. The organism is gram negative.

CULTURAL CHARACTERS

The organism grows well on potato agar with 1 or 2 per cent dextrose and no peptone, and this medium has been generally used. Equally good growth occurs on lima-bean agar without dextrose. Unless otherwise specified, the cultures were incubated at room temperature. The reaction of the media as expressed in Fuller's scale was adjusted by titration, using as the neutral point the first permanent but faint pink color with phenolphthalein. Inoculations were made from water suspensions unless otherwise stated. Cultures of *Bacillus coli* and *Bacterium glycineum* Coerper were carried in parallel series in part of the tests.

AGAR POURED PLATES.—On + 10 beef-peptone agar, colonies appeared in 24 hours; and in 56 hours surface colonies were 2 mm. in diameter, and round, raised, glistening, and grayish white. Submerged colonies were smaller and lens shaped. In six days surface colonies were round with an entire margin, raised, and smooth, with a finely granular internal structure showing faint concentric markings (pl. 4, A). The color was grayish white in reflected light and slightly fluorescent in transmitted light.

Surface colonies on potato-dextrose agar incubated four days at 27° C., were 3 to 4 mm. in diameter, and in one week were 5 to 7 mm. in diameter. The colonies were round, and the margin entire (pl. 4, A); the surface was smooth or concentrically ridged; and the elevation varied from raised to pulvinate, with the central portion often higher, making the colony umbonate. The colonies had a finely granular appearance, with a more or less concentric pattern, as

illustrated in Plate 4, C, and were grayish white in reflected light and greenish fluorescent in transmitted light. The colonies which emerge after the agar surface has dried slightly seem to pile up more conspicuously. Colonies on agar that has dried out to some extent have a scalloped margin and sculptured surface, as shown in Plate 4, B. Submerged colonies are lens-shaped, white, and very small (pl. 4, A). Colonies on the underside of the agar are thin, transparent, and greenish fluorescent. The agar is unchanged in color. Only a slight odor is noticeable.

AGAR STABS.—In +10 beef-peptone agar, growth along the stab was scanty and filiform, and on the surface was restricted but piled up. In potato-dextrose agar, growth occurred in the stab along the upper part only, and this was slight; but on the surface the growth was abundant, spreading, flat, and dull with a rugose zone. Growth on this medium was much more vigorous than on the beef-peptone agar.

AGAR SLANTS.—On +10 beef-peptone agar, the growth was moderate, spreading, flat, smooth, and grayish white. On potato-dextrose agar the growth was abundant, spreading, flat, dull, finely rugose, and grayish white. The margin was entire, with a definite beveled border. There was no change in the color of this medium in either case. On lima-bean agar a slight greenish pigmentation of the medium occurred.

GELATIN PLATES.—In two days flat, circular, white colonies, producing saucer-shaped zones of liquefaction, were present. Liquefaction had proceeded to completion the third day.

GELATIN STABS.—The liquefaction was napiform in 2 days, infundibuliform in 3 days, stratiform in 7 days, and complete in 14 days, with a white flocculent precipitate.

POTATO CYLINDERS.—On steamed potato cylinders growth was rapid and abundant, grayish white, and somewhat iridescent, smooth, and glistening, and had spread over all the moist surface of the substratum. There was no change in the color of the potato tissue.

MILK.—Clearing of the milk without coagulation began at the top in 2 days and was complete in 33 days. Throughout this period the cleared liquid was of a pale greenish-yellow color, and the liquid became viscid or gelatinous in consistency.

LITMUS MILK.—Pale-blue litmus milk was completely decolorized in seven days, but no pink color appeared. Digestion proceeded as noted above and was complete in 6 days. The cleared liquid was slightly yellowish green.

BROM CRESOL PURPLE IN MILK.—Brom cresol purple produces a light bluish color in milk and becomes yellow if the hydrogen-ion concentration is increased. Seven days after inoculation the color was unchanged, except that it was even more purplish in the upper cleared portion. After 21 days the blue color remained. Accordingly, there is no acid production in milk.

METHYLENE BLUE IN MILK.—In this medium a deeper blue color was noted at the end of two days, while after seven days the milk was completely decolorized except for the cleared portion at the surface, which was greenish. After 33 days the color was maize yellow, except for a thin greenish layer at the surface.

REDUCTION OF NITRATES.—In fermentation tubes containing 1 per cent potassium nitrate in a 2 per cent Difco peptone solution, there was good growth in the open arm and none in the closed arm. No gas was formed. At the end of a month the liquid in the open arm showed a slightly yellowish green. Test-tube cultures in the same medium were tested with Trommsdorf's reagent at 14 and 33 days after inoculation, and no nitrites were detected. With Nessler's reagent, a strong positive test for ammonia was obtained. Apparently nitrates were not reduced, and ammonia probably was produced from the peptone.

CARBON METABOLISM.—To test for acid and gas production with different carbon sources, 2 per cent solutions of dextrose, saccharose, maltose, lactose, mannite, and glycerin were made up in a 2 per cent Difco peptone solution. Cultures were run in duplicate, first in the ordinary U type of fermentation tube and later in a simpler and more convenient type of fermentation tube (*9, fig. 47*) consisting of a smaller inverted test tube within a larger one, a type very satisfactory where it is not necessary to measure the quantity of gas. In all cases there was abundant growth in the open arm and none in the closed arm. No gas was formed. Titration with N/20 sodium hydroxide at the end of 17 days in one series and 33 days in the other revealed no marked change in acidity as compared with the sterile control tubes. The lactose and glycerin cultures were slightly less acid than the controls in one series.

In order to determine more accurately the changes in true acidity in these media, three series of six test tubes each were made up, one series containing brom cresol purple, one brom thymol blue, and the other phenol red in a concentration of 0.0016 per cent. These media were adjusted to about P_H 7.3 as indicated by the blue-green color of brom thymol blue and the red of the phenol red. The dextrose and saccharose cultures with brom thymol blue and phenol red became yellow (more acid) seven days after inoculation. All the other cultures slowly became less acid than the controls. There was, therefore, acid production from dextrose and saccharose, but none from the other carbon sources. Probably the decrease in true acidity was due to ammonia produced from the peptone.

AGAR WITH SUGARS.—In litmus-dextrose, litmus-maltose, and litmus-lactose agar slant cultures there was no evidence of acid production. In two days the maltose and lactose cultures were blue under the stroke, and in seven days all cultures were bluer throughout than the controls. However, in a later repetition of this series, in which saccharose, mannite, and glycerin were also tested, a pink color developed in the dextrose and saccharose tubes, indicating acid production.

To obtain more accurate information on this point, the three sulphone phthalein indicators, brom cresol purple, brom thymol blue, and phenol red, were used in a triplicate series of slant cultures with dextrose, maltose, and lactose. These media were adjusted to a P_H of about 7.0 as evidenced by the grass-green color of brom thymol blue. In the dextrose cultures containing brom cresol purple and brom thymol blue a yellow color developed, indicating acid production. No increase of hydrogen-ion concentration was indicated at any time with the other two sugars and most of the cultures became slightly more alkaline. In a later repetition of this test, saccharose, mannite, and glycerin were also included and distinct acid production occurred only in the dextrose and saccharose cultures. Parallel series of cultures of *Bacillus coli* produced the yellow-acid color with all of the indicators. These tests show that there is acid production from dextrose and saccharose.

ACTION ON STARCH.—There were no signs of diastatic action on either potato or corn starch. No halos or cleared zones appeared about the colonies in plates of beef agar to which starch was added.

TESTS FOR INDOL, SKATOL, AND AMMONIA.—Cultures in beef-peptone bouillon gave no test for indol at 7, 26, and 33 day intervals when tested with potassium nitrite and sulphuric acid and no test for skatol when tested with nitric acid and potassium nitrite. A positive test for ammonia was obtained after 7 and 33 days with Nessler's reagent.

FERMI'S SOLUTION.—Growth occurred in Fermi's solution, accompanied by the formation of a greenish-yellow pigment throughout the medium.

USCHINSKY'S SOLUTION.—Good growth occurred in Uschinsky's solution in the case of two of the three strains tested, and a greenish-yellow pigment was produced.

COHN'S SOLUTION.—No growth occurred in Cohn's solution.

BLOOD SERUM.—The growth in stroke cultures on plain solidified blood serum in 10 days was abundant, spreading, flat, smooth, and glistening, and showed a brownish tinge. A slight liquefaction of the medium along the stroke was noted after 17 days, and at the end of 40 days there was a general liquefaction of the upper part of the slant, and a brownish-yellow discoloration of the medium.

On slants of Loeffler's blood serum, growth was more rapid than on plain blood serum, and in two days was abundant, spreading, flat, and rugose. In 10 days the medium showed a slight brownish color and slight liquefaction under the stroke. At the end of 40 days the medium was almost completely liquefied, and was clay color according to Ridgway's chart (*16*).

TOLERATION OF SODIUM CHLORIDE.—In tubes of beef-peptone bouillon neutral to brom thymol blue, 4 per cent of sodium chloride was tolerated and 5 per cent inhibited growth. In a series neutral to phenolphthalein, 5 per cent of sodium chloride was tolerated. Evidence was obtained that the use of hydrochloric acid in adjusting the reaction increased the inhibitory properties of sodium chloride.

TOLERATION OF ACIDS AND ALKALIES.—Tubes of beef-peptone bouillon were adjusted to +30, +25, +20, +18, +15, +14, +12, +10, +5, 0, −5, −10, −15, −20, −30, and −40 Fuller's scale by the use of hydrochloric acid and sodium hydroxide. The three strains grew in the +10, +5, 0, −10, and −15 tubes, and two strains grew in the +12 tubes. In a later series growth occurred in the +15 and −25 tubes. Growth seemed most vigorous in the +5 media. A greenish-yellow pigment was formed in the alkaline cultures.

In order to determine the tolerance of true acidity as indicated by the hydrogen-ion concentration, a duplicate series of beef-broth tubes was made up, one with hydrochloric acid and the other with malic acid, and adjusted by means of the sulphone phthalein indicators. No growth occurred in the tubes acidified to P_H 4.1 with either acid, but retarded growth occurred in both cases in the tubes acidified to P_H 4.5. Good growth occurred in the P_H 5.0 tubes. Growth occurred in tubes rendered alkaline to P_H 8.5 with either sodium hydroxide or ammonium hydroxide.

TEMPERATURE RELATIONS

The organism grows throughout a wide range of temperatures. Slant and plate cultures on potato agar incubated in moist chambers at 3° C., 9°, 12°, 15°, 20°, 23°, 27°, 30°, and 35° showed that the organism did not grow at 3° or 35°, but grew slowly at 9°, and 12°, moderately at 15°, 20°, and 35°, and rapidly at 23°, 27°, and 30°, with a fairly distinct optimum at 27°.

In determining the thermal death point, water suspensions from agar slant cultures were subjected to 10-minute exposures to a series of temperatures in a water bath and tested by loop transfers to agar slants (*12*). The thermal death-point was found to lie between 49° and 50° C.

EFFECT OF FREEZING

Heavy water suspensions from slant cultures were placed in test tubes, frozen in an ice-salt mixture, and held in a refrigerator. At intervals tubes were removed and thawed out and plates poured. The approximate number of living bacteria per cubic centimeter as indicated by the plate counts is shown in Table I.

TABLE I.—*Effect on bacteria of freezing in water*

Time frozen	Approximate number per cubic centimeter		Time frozen	Approximate number per cubic centimeter	
	Strain A	Strain B		Strain A	Strain B
0 *	12,635,000	106,714,000	5 days	2,000	149,000
4½ hours	3,192,000	35,285,000	8 days	250	2,400
1 day	2,777,000	13,050,000	11 days	360	540
2 days	27,000	1,224,000	15 days	20	0
4 days	4,000	21,000			

* Original suspension.

The results in Table I show that the organism is slowly killed by freezing in water.

EFFECT OF SUNLIGHT

Plates poured from tubes inoculated with a suspension of the organisms, and each partly shaded with black paper attached to the glass, were placed on a cake of ice and exposed to the afternoon sun for periods of varying length. Ten and fifteen minute exposures did not reduce the number of colonies, while 25, 30, and 45 minute exposures greatly reduced the number of colonies, and exposures of 60 minutes or more resulted in complete sterilization.

RESISTANCE TO DESICCATION

The organism is very sensitive to desiccation on glass. Drops of a water suspension of the organisms were allowed to dry on sterile cover slips, and tests were made by inserting the cover slips into agar

slants. No growth was obtained from smears that had been dry 40 minutes, and in most instances no growth was obtained from smears that had just dried.

To test the resistance of the organism to drying on cowpea seeds, small quantities of seed were placed in Petri plates, moistened, sterilized in the autoclave, and allowed to become air-dry. A water suspension of the organisms was then poured over this sterilized seed and allowed to dry. The seed was tested at intervals by planting in agar-poured plates and the organisms were found alive during the following four months. Furthermore, it has been found that the organism lives over winter in the seed, so that it is evident that it is highly resistant to drying on and in cowpea seeds.

Tisdale and Williamson (*24, p. 150*) found the organism alive in lima-bean leaves dried 2½ years. In the writers' experience the organism has not generally been found viable in the older tan-centered leaf lesions on cowpeas; it has a tendency to be rather short lived on potato-agar slants, much more short lived than certain yellow organisms such as *Bacterium phaseoli* E. F. S. and *Bact. vesicatorium* Doidge.

TAXONOMY

The organism causing bacterial spot of cowpea is not identical with any of the previously described bacterial parasites of cowpea, being clearly differentiated in its chromogenesis and other salient characters. It was briefly described in March, 1923, (*6*), and given the name *Bacterium vignae* n. sp. Upon the appearance of this preliminary note the writers received a letter from W. B. Tisdale calling attention to the marked similarity of this organism to the causal organism of the lima-bean disease, a description of which was in press at that time and appeared four months later (*24*). The causal organism of the latter disease was designated *Bacterium viridifaciens* n. sp.

The lima-bean disease was found rather commonly in gardens about La Fayette, Ind. The causal organism was isolated from pod lesions and proved to resemble closely the cowpea organism. With the organism isolated from lima beans, abundant and typical infection of cowpea seedlings grown under a cloth cage in the field was obtained August 15, 1923, along with infection of lima-bean seedlings. In another cage, typical infection of lima-bean seedlings was obtained with one of the strains isolated from cowpeas. Later a culture of Tisdale's organism was obtained from the University of Wisconsin, and with it successful inoculations of cowpea seedlings with the production of characteristic bacterial spot lesions were obtained in the greenhouse in April, 1924. These cross inoculations indicated beyond doubt the identity of the two organisms.

In the meantime, however, the cowpea strain, the strain isolated from lima beans at La Fayette, Ind., and the original lima-bean strain from the Wisconsin laboratory were carefully compared as to a large number of cultural and physiological characters and found practically identical. These tests included the ordinary media such as gelatin, agars, and milk with indicators, agar and bouillon with the six carbon sources and the three hydrogen-ion indicators previously mentioned, toleration of sodium chloride, toleration of acidity, blood media, and Cohn's, Fermi's, and Uschinsky's media. Furthermore, the published description of *Bacterium viridifaciens*

is practically in complete accord with the writers' earlier description and the data herein recorded. While the writers failed to detect an increase in acidity in the dextrose and saccharose fermentation-tube cultures by titration, they obtained striking evidence of acid production with these sugars in the cultures containing the hydrogen-ion indicators. The only difference in group number is due to the writers' failure to class the organism as fluorescent. Since the cowpea and lima-bean organisms are thus shown to be identical, the binomial *Bacterium viridifaciens* becomes synonymous by priority rules with the previously published name, *Bacterium vignae*.[4] (Group Number, 5322–31131–2232).

This organism shows some resemblance to certain other plant pathogenes, such as *Pseudomonas pisi* Sackett (*17*), which it resembles culturally but from which it differs in morphology and pathogenicity, and *Pseudomonas maculicolum* McCulloch (*13*), which it resembles culturally and in type of lesion produced but from which it differs in pathogenicity. It differs both in culture and in pathogenicity from *Bacterium glycineum* Coerper, *Bact. trifoliorum* Jones, Williamson, Wolf, and McCulloch, and *Bact. tabacum* Wolf and Foster, although the Virginia strain of *Bact. trifoliorum* was found to be pathogenic to lima bean and velvet bean (*11, p. 486*). The organism differs radically in morphology from *Aplanobacter stizolobii* Wolf (*26*), the causal organism of bacterial leaf spot of the velvet bean. Attempts to infect cowpeas with *Pseudomonas pisi* have given only negative results, corroborating Sackett's (*17, p. 18*) conclusion that cowpeas were not a host for that organism.

PATHOGENICITY OF CAUSAL ORGANISM

Infection of young, healthy cowpea plants has been produced at will by spraying (from an atomizer) with a water suspension of a young agar slant culture. This has been done in the field and more frequently in the greenhouse, where the plants could be held in a moist chamber for one or two days after the inoculation. The same has been done with young lima-bean plants.

The writers have found, as did Tisdale and Williamson (*24*), that the organism tends to lose its virulence in culture rather rapidly and that best results are obtained with recently isolated or reisolated strains.

The incubation period for leaf infection of cowpeas and lima beans under greenhouse conditions is two to four days, and in the field in August lesions have become visible two days after inoculation. Wounds are not necessary for infection, and the abundance of lesions indicates that the mode of entry into the host tissue is undoubtedly stomatal.

Cowpea seedlings and the younger leaves of older plants have proved much more susceptible to infection than older parts of the plant. It seems possible that this fact may be correlated with a lower hydrogen-ion concentration in the young leaves, a condition found to exist in clover by Haas (*8, p. 350*) and later in pole beans by Gustafson (*7*). In the writers' work with bacterial spot of

[4] According to Migula's classification, Buchanan's revision (*2, p. 48*), and the revision adopted by the committee of the Society of American Bacteriologists (*21, p. 203*), the combination would be *Pseudomonas vignae* n. sp., while in a later report of another committee of the same society (*22, p. 188*), the name has already been changed to *Phytomonas vignae*.

tomato (*5, p. 148*) a correlation was found between the resistance of ripe fruit to infection and the higher hydrogen-ion concentration in the ripe fruit as compared with the leaves and green fruit, and in that disease also the younger parts of the plant were more susceptible to infection.

Clevenger (*4, p. 238*) found that the hydrogen-ion concentration of cowpea leaves was slightly lower than in the stems, and that it showed a diurnal variation (*4, p. 233*). A maximum concentration (P_H 5.27) occurred in the leaves at 10.30 a. m. and a minimum (P_H 5.81) at 1 a. m., while in the stems a maximum (P_H 5.04) occurred at 6.30 a. m. and a minimum (P_H 5.32) at 9 p. m. The writers in their cultural tests found that the parasite tolerated a P_H of 4.5 and grew well at P_H 5.00, so it is evident that the cowpea leaves and stems are well within the limit of tolerance. The lower acidity of the leaves in the night may favor infection at that time.

Atomizer inoculation has been successful upon all of the varieties of cowpea, sieva bean, and lima bean tested, including most of the varieties mentioned in the previous discussion of hosts of this disease, and upon the catjang, hyacinth bean, adsuki bean, and Florida velvet bean (varieties, Bunch and 100-Day). The organism was successfully reisolated from each host species. The three *Phaseolus limensis* varieties—Burpee, Fordhook, and Large White Pole—have appeared to be more susceptible than Henderson's Bush Lima, which, according to Bailey (*1, p. 396*), is a different species. In fact, in the writers' field plots in 1924, Henderson's Bush Lima showed considerable resistance. While the cowpea varieties have been considered equally susceptible, the varieties Early Red, Clay, California Blackeye, Iron, Groit, and Whippoorwill were more severely diseased than any of the other 12 varieties in the 1923 plots. In the 1924 plots the varieties Early Red, Groit, Whippoorwill, New Era, and Catjang were more severely diseased than Blackeye, Iron, and Early Buff. Rather light natural infection occurred on the foliage of velvet beans grown adjacent to cowpeas in 1924, and the organism isolated resembled *Bacterium vignae* in culture, and in inoculation tests produced typical lesions on cowpeas.

The organism isolated from the lesions on *Desmodium canescens* resembled the cowpea organism in culture, and in cross-inoculation tests in a field cage and in the greenhouse produced typical bacterial spot infection on cowpeas. The organism was successfully reisolated, and although its morphology and cultural characters have not yet been studied by the writers, it seems safe to assume that the Desmodium organism is *Bacterium vignae*. The importance of this weed as a possible source of infection is not known.

Unsuccessful attempts have been made to inoculate garden beans (five varieties), soy beans (five varieties), broad bean, sweet pea, peas (seven varieties), lupine, clovers (white, Bokhara, crimson, annual sweet, mammoth red, and alsike), cauliflower, tobacco, tomato, and potato.

RELATION OF PARASITE TO HOST TISSUE

As atomizer inoculation of cowpeas without wounds is successful, it seems likely that the tissues are entered by way of the stomata. Tisdale and Williamson (*24, p. 151*) found that the lima-bean leaf was invaded through the stomata. Microscopic examination of the

leaf lesions shows that the invasion is intercellular and tends to be restricted to the mesophyll during the early stages. In fact the mesophyll may be rather extensively involved without any apparent injury to the palisade tissue. The advance invasion is in the mesophyll layers adjacent to the lower epidermis. The cells involved become a dense reddish brown and soon collapse. The lower mesophyll layers may thus become discolored and collapsed while the upper mesophyll layers and the palisade layer remain apparently uninjured. Later the entire thickness of the lamina is killed and dries out at the center of the lesion.

In cowpea seedlings vascular infection has been frequently observed. The organism may gain entrance to the vascular system from infected cotyledons, epicotyl lesions, petiole lesions, or from lesions on the veins of the leaf. Reddish-brown bundles were traced from the infected cotyledon or epicotyl down into the hypocotyl and up through the epicotyl into the leaf veins (pl. 4, F). In water mounts, the bacteria were seen to ooze from the cut ends of these reddish-brown spiral vessels of the epicotyl, and in cross section these vessels appeared to contain bacteria. The organism was cultured from these internally discolored vascular bundles at some distance from the lesion where it had entered the host. From lesions on the larger veins of the seedling leaves, vascular infection may extend outward a distance of a centimeter or more, and in some cases the reddish-brown vascular elements, being in the xylem, are more clearly visible from the upper leaf surface.

A microscopic examination of unstained razor sections of these internally discolored veins showed that the reddish-brown color was localized in the walls of certain spiral vessels and that this color was very intense, constituting an excellent stain for the walls of the vessels even under the higher powers of the microscope. The stain was also noted in the tubes of the small veinlets in the affected areas. Stained strips of collapsed cells also occurred in the dorsal cortex of the veins and in the mesophyll along the veins.

When the freshly cut edges of leaf lesions were examined in water mounts, the bacteria were seen to ooze from the cut ends of the veins. In cross section the bacteria were noted in the intercellular spaces of the parenchyma of the veins but were not actually demonstrated within the spiral tubes at any distance from the leaf lesion. The organism very evidently shows a preference for the vein tissues and travels more rapidly in the veins than in the mesophyll. In older leaves the tendency for internal vascular invasion is not as marked, although linear surface lesions of considerable length often occur along the veins (pl. 1, A, B; pl. 2, D).

Shallow surface lesions on the epicotyl, hypocotyl, and petioles were found to be limited to the outer cortical layers. The cells in the epidermal and cortical layers were collapsed and dense dark red in color. In large hypocotyl lesions there was evidence of deeper invasion, not in a solid front, but in the shape of ramifying intercellular penetration. There was marked hypertrophy of the cortical cells immediately beneath the epicotyl lesions, and in many cases rather extensive hyperplasia had resulted from the formation of cross walls in these enlarged cells, suggestive of an attempt to occlude the lesion with a cork layer. Very early infection of the

epicotyl results in linear lesions occupying deep grooves and in cross section it was seen that these lesions extended inward through the cambium to the pith and were accompanied by extensive hyperplasia of the latter tissue. These lesions result in a break or uncompleted segment in the vascular cylinder, and, as a result of enlargement of the rest of the epicotyl tissues, become sharply sunken channels or grooves. Shallower lesions were noted which had not interrupted or impaired the cambium and which had occasioned a marked hyperplastic response.

TRANSMISSION OF THE DISEASE THROUGH SEED

Owing to the abundance of cowpea-pod lesions, many of which were found to penetrate through the pod tissues into the seed, actual infection of the seed by this organism may occur very readily and very generally (pl. 3, C and E). Furthermore, it was found that the organism could endure long periods of desiccation on the surface of the seed, being found viable after four months of drying.

In order to determine the possibility of seed transmission of the disease, a number of seeds selected from diseased pods collected in the fall of 1921 were sown in pots of sterilized soil in the greenhouse on March 8, 1922. Among 123 seedlings grown from seeds borne directly under pod lesions, 18 showed bacterial spot infection; and among 198 seedlings grown from seeds not borne directly under lesions, 5 showed infection. These tests proved that the disease was carried with the seed and that infected or contaminated seed gave rise to diseased seedlings.

This primary infection consisted of lesions on the hypocotyl, epicotyl, cotyledons, and first leaves. In some cases lesions had originated at the point of attachment of a cotyledon and extended down into the hypocotyl (pl. 3, F) and up into the epicotyl. Infected cotyledons tended to remain attached longer than normal ones. In one case there was one lesion on each first leaf, each located on the under side of the midrib at a point corresponding exactly with the location of the other. Considerable local vascular infection and localized wilting occurred among these seedlings. The organism was successfully isolated from these seedlings.

Similar tests in pots of sterilized soil were made with 20 lots of commercial seed from two sources representing 16 varieties. The shriveled, discolored seeds were tested separately. The seed was planted April 26, 1922, and the results as noted on May 12 are given in Table II.

The results given in Table II show that the infection was present in 15 of the 20 commercial seed lots tested, and occurred in the seed from both sources and in seed of normal appearance as well as in shriveled or discolored seed. Most of the infected plants represented primary seed-borne infection. The organism was isolated from a number of the seedlings. The high incidence of infection in some of the seed lots, coupled with the large proportion of the lots showing infection, indicates the great extent to which the disease may be present in cowpea seed. The appearance of a seed can not be depended upon to indicate whether or not it may transmit the disease.

TABLE II.—*Tests of commercial cowpea seed samples for the presence of bacterial spot infection*

Variety	Source [a]	Seeds normal in appearance		Seeds shriveled or discolored	
		Number of plants	Number infected	Number of plants	Number infected
Early Red	A	14	0	32	0
Early Black	A	19	0	22	2
Early Buff	A	28	0	32	2
Conch	A	12	0	20	0
Taylor	A	23	0	35	1
Brabham	A	28	1	37	0
California Blackeye	A	23	16	24	11
Vigna catjang	A	18	0	34	2
New Era	A	28	1	36	4
Do	B	13	0	19	0
Whippoorwill	A	30	0	24	4
Do	B	21	0	16	0
Iron	A	19	4	31	10
Do	B	20	0	23	0
Groit	A	29	7	35	0
Do	B	17	0	14	0
Red Ripper	B	21	0	29	2
Black	B	24	0	21	1
Wonderful	B	28	0	30	6
Clay	B	26	6	23	3

[a] Two sources designated A and B.

To determine the effect of age of the seed upon the presence of bacterial spot infection, 125 plants were grown in sterilized soil in the greenhouse in March, 1923, from the same lot of seed of the Whippoorwill variety with which the experimental plot had been planted in 1921. The disease had appeared in this field plot very evidently as a result of seed-borne infection, but did not appear on the greenhouse plants grown in 1923. This would indicate that the extra two years of storage had eliminated the organisms.

TABLE III.—*Effect of one extra year of storage on bacterial spot disease transmission in infected seed in certain commercial seed lots listed in Table II, determined by planting some of the seed in sterilized soil in the greenhouse in March, 1923*

Variety	Source of seed [a]	Planted April, 1922		Planted March, 1923	
		Number of plants	Number infected	Number of plants	Number infected
Early Black	A	41	2	66	0
Early Buff	A	60	2	37	0
Vigna catjang	A	52	2	131	0
New Era	A	64	5	63	0
Iron	A	50	14	35	0
Groit	A	64	7	89	0
Red Ripper	B	50	2	19	0
Wonderful	B	58	6	29	0
Clay	B	49	9	23	0

[a] Two sources designated A and B.

The results in Table III indicate that one year of storage had eliminated the seed infection in every variety tested. While the age of this seed is not known with certainty, it is very probable that it came from the 1921 crop. Although these pot tests represent only a small number of seed, it would seem that seed two and three years old is freer from infection than one-year-old seed, as was found by Rapp (*15*) in the case of bean bacterial blight.

CONTROL

Since the disease is seed borne in the case of cowpeas, prevention of it would primarily require the use of disease-free seed. Disease-free seed can be obtained in small quantities by seed selection from disease-free pods. The use of two or three year old seed should result in decreased primary infection.

In 1924 the disease was found on volunteer plants in a plot that was in cowpeas the year before. Therefore crop rotation would seem advisable.

SUMMARY

Bacterial spot of cowpea is characterized by reddish-brown lesions on the leaves, stems, pods, and seeds.

The hosts include all varieties of cowpea tested, catjang, hyacinth bean, asparagus bean, adsuki bean, velvet bean, lima bean, bush lima bean, dwarf sieva bean, and the common weed, tick trefoil. Henderson's Bush Lima bean has shown some resistance.

On cowpeas the young growing organs are most susceptible, and infection of such organs results in considerable distortion and shattering of the leaves, deformity of the pods, and discoloration and stunting of the seeds. In seedlings and young leaves localized vascular infection and localized wilting may occur.

The causal organism is a motile rod bearing one to five flagella at one or both poles, first described as *Bacterium vignae* n. sp. It is identical with *Bacterium viridifaciens* n. sp. described by Tisdale and Williamson.

The colonies on agar are grayish white and, in transmitted light, slightly fluorescent. Gelatin is liquefied.

Acid is produced only with dextrose and saccharose. Greenish pigment formation occurs in milk, alkaline broth, and in Fermi's and Uschinsky's solutions.

Atomizer inoculation without wounds is successful. The invasion in cowpea leaves is intercellular, and the mesophyll is most extensively involved. Vein tissues are preferred and vascular invasion may occur in seedlings and young leaves. Epicotyl lesions may be accompanied by hypertrophy and hyperplasia of the underlying cortical or pith cells.

Cowpea-pod infection results in seed infection. Seeds from infected pods gave rise to infected seedlings when planted in sterilized soil. Commercial seed of a number of cowpea varieties planted in sterilized soil also gave rise to infected seedlings. Similar seed stored an extra year seemed to be comparatively free from infection.

As control measures with cowpeas, the selection of seed from disease-free pods, or the use of seed two or three years old, and crop rotation are suggested.

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THE INFLUENCE OF CERTAIN ENVIRONMENTAL AND CULTURAL CONDITIONS ON FRUIT-BUD FORMATION OF PEAR AND APRICOT[1]

By C. B. WIGGANS[2]

Department of Horticulture, University of Arkansas

INTRODUCTION

The pear and apricot fruits are formed from flower buds differentiated during the summer previous to blossoming. It is generally assumed that fruit-bud formation can be influenced only by operations performed before the time of differentiation. If this is true, the orchardist is not only interested in the approximate time of differentiation, but also in the influence which certain cultural operations, such as pruning and irrigation, may have on the time and subsequent development of fruit buds. To add to the present knowledge of flower-bud formation, and to obtain information which might be of immediate practical value to the fruit industry of California, an attempt has been made to answer the following question: What influence do (1) different degrees of severity of dormant pruning, (2) irrigation, and (3) climatic conditions in the coastal valleys, interior valleys, and foothills have upon the time of differentiation and the rate of development of the fruit buds of the pear and apricot?

As far as the writer knows, the only exact data which had been collected with regard to fruit-bud formation in California previous to the present investigation were those that dealt with average interior-valley conditions, no attempt having been made to determine the influence that such cultural practices as pruning and irrigation might have upon the time of differentiation and subsequent development of fruit buds. Likewise, data collected in the Eastern and Northwestern States possibly do not apply directly to California conditions, and furthermore, these other investigations have devoted little attention to the special problems involved here, since irrigation with them is not a common practice and neither does there exist such a diversity of pruning methods as in California.

Pruning, commonly recognized as a necessary orchard operation, is one of the most expensive and time-consuming practices of the California orchardist. In this State there are practically as many pruning methods as fruit-growing sections. Until recently the common practice in California has been to severely head back at the dormant pruning. A radically different method is rapidly gaining favor. This system consists essentially of thinning-out only and is popularly known as "long" pruning as opposed to the method of heavy heading-back or "short" pruning.

[1] Received for publication Nov. 28, 1924; issued January, 1925. The work was carried on under the auspices of the California Agricultural Experiment Station.

[2] The writer wishes to express his appreciation to the following: Prof. W. P. Tufts for counsel and suggestions in planning and carrying forward this investigation; Dr. W. W. Robbins and Dr. W. L. Howard for suggestions and criticisms on the preparation of the manuscript; Miss Edna Russ for her assistance in the preparation of the photographs; and B. J. Jones and Henry P. Everett for collecting part of the material.

Because of the comparatively light rainfall in winter and complete absence of rain in summer, irrigation is, with the exception of a few limited areas, a necessity for successful orcharding in California.

The problem of fruit-bud formation in California is further complicated by the fact that here latitude means little or nothing in determining climate. Isotherms normally run east and west. In California, with the high Sierra Nevada mountains on the east, the Pacific Ocean on the west, and minor mountain ranges on the north, south, and west, these isotherms are deflected so that their general direction is north and south (fig. 1).

In California there are three distinct climatic belts differing in degree rather than kind. These are coast, valley, and mountain, depending on distance from the ocean, location with reference to

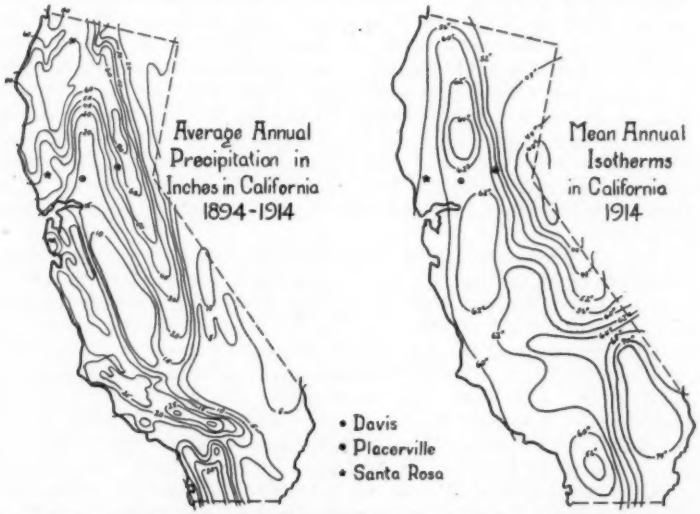


FIG. 1.—Outline map of California, showing average annual precipitation in inches for 20-year period 1894–1924; isotherms for mean annual temperatures in 1914; also the points from which fruit buds were collected

mountain chains, and altitude for their climatic differences. Fruit-growing sections are found in all of these belts.

The fruit-growing districts of the Pacific coast are located in protected places just back of low mountains, and are designated as coastal valleys. As compared with the interior valleys, these coastal valleys are characterized by warmer winters, cooler summers, more abundant winter rainfall, slightly more humidity in summer, and frequent fogs.

The interior valleys have the following general climatic characteristics: Higher summer and lower winter temperatures than the coastal valleys, abundant winter rainfall in the north decreasing rapidly southward; very dry air, free from fogs in the summer and with almost constant sunshine; occasional strong, hot, desiccating winds from the north in summer, and cold north winds in winter.

On the way up the foothills surrounding the valleys the seasons become increasingly distinct, mildly cold winters and warm summers being experienced. Hardy apples and pears grow well up to 4,000 feet elevation or somewhat higher. However, the chief fruit-growing sections of the mountains are found in the foothills at elevations of not higher than 2,500 feet. The climate in the foothills is a modification of the interior-valley climate. Temperatures are slightly lower, and the rainfall increases at the rate of about 1 inch to each 100 feet of elevation (fig. 1).

MATERIAL

The pear and apricot were chosen for study. These fruits were selected not only because of their commercial importance in California, but because one was a pome and the other a drupe. It was thought that other fruits of these classes might tend to be influenced in much the same way even though differentiating at very different times.

Pear and apricot buds were collected from three typical fruit-growing districts, namely: Sonoma County north and east of Santa Rosa, as representative of coastal valley climatic conditions; university farm at Davis, Yolo County, as representative of interior-valley climatic conditions; and Eldorado County east of Placerville, as representative of foothill climatic conditions.

Buds were collected in Sonoma County from Bartlett pear trees growing under three cultural conditions—heavily pruned and irrigated, heavily pruned and nonirrigated, and lightly pruned and nonirrigated. The trees were typical, heavily pruned specimens in good health, growing on Dublin loam, and were about 20 years old. The nonirrigated and lightly pruned tree was in good health, 8 or 10 years old, and was growing on yellow clay loam.

Bartlett pear trees growing on the unviersity farm, Yolo County, under these three conditions were compared: Lightly pruned and irrigated, heavily pruned and irrigated, lightly pruned and nonirrigated. The first two mentioned were within 50 feet of each other and growing under apparently identical field conditions, except for pruning treatment. The soil was a Yolo sandy loam. The heavily pruned tree was typical, but the lightly pruned one was perhaps more vigorous than the average tree thus pruned. The lightly pruned and nonirrigated tree was located about one-quarter mile from the others, on Yolo clay loam, but under much the same conditions except for irrigation. The trees were all 7 years of age, and showed vigorous growth.

Collections of buds were made from only one tree in Eldorado County, at an elevation of approximately 2,400 feet. The tree was lightly pruned and irrigated, 7 or 8 years of age, and in a vigorous condition.

Apricots are not widely grown in Sonoma and Eldorado Counties, and it proved difficult to find suitable examples of this fruit under coastal valley and foothill conditions. However, collections were taken from the best trees that could be found in these locations.

The Royal apricot tree chosen in Sonoma County was 10 or 12 years old, lightly pruned and nonirrigated, and grew on a gravelly, fine, sandy loam.

Buds from Royal apricot trees growing under the three following conditions on the university farm were compared: Lightly pruned and nonirrigated, heavily pruned and irrigated, lightly pruned and irrigated. Each tree grew near and under the same cultural treatment as the pears described above. All were typical except the lightly pruned and irrigated tree, which apparently lacked vigor.

Few apricot trees could be found at the relatively high elevation in Eldorado County. The tree selected was thought to be a specimen of the Royal variety. It was an aged tree growing at an approximate elevation of 2,200 feet, and had received little or no pruning and but limited irrigation.

All trees in the experiment were under clean culture, with the exception of the apricot last mentioned.

Collections were made on or near July 10 and 20; August 1, 10, and 20; September 1 and 15; October 1 and 15; and November 1 from all trees except those in Eldorado County, where there were none taken July 10. In addition to these, samples were taken from all the trees at the university farm on December 1, and from the lightly pruned and irrigated pear tree and from the heavily pruned and irrigated apricot tree at intervals of from one to two weeks from January 1 until blossoming time.

METHODS

All buds were collected from fruit spurs not more than 3 inches long, and from all sides of the tree. About 30 buds were taken from each tree at each collection. Since fruit buds only were desired, the largest, plumpest pear buds and double and triple collateral buds of the apricot were taken. In the case of the apricot, contrary to the published inferences of Gourley (*8*),[3] Bailey (*1 p. 38*) and others, the middle bud is not always a leaf bud. Many of these collateral buds were sectioned as a unit and all found to be flower buds.

Upon removal from the tree, the buds were immediately put in a formalin-alcohol killing and fixing solution, in which they were kept until ready to use. In later collections the outside scales were removed before killing, and with pears even the top part of the individual flower buds was cut away to eliminate the abundant hairs which hung on the knife edge of the microtome and prevented the proper ribboning of the paraffin mounts.

The major part of the sectioning was accomplished by the paraffin method as described by Chamberlain (*2*). The outstanding departures from Chamberlain's methods were less gradual dehydration and the holding of the material in the alcohol solutions for a shorter time. Safranin dissolved in alcohol and gentian-violet in clove oil proved to be the most satisfactory combination stain. Light green with safranin and Delafield's haematoxylin with safranin were tried but found less useful. Haidenhain's iron-alum haematoxylin proved to be useful as a nuclear stain but difficult to handle. Coplin jar tops, inverted and the inside surface coated with a thin film of glycerine, proved useful as embedding molds. Sections from 5 to 12.5 microns in thickness were cut. The thicker sections proved more satisfactory for photographing gross morphology. The thinner

[3] Reference is made by number (italic) to "Literature cited," p. 882.

sections were made for the cytological study of pollen and ovary development. Only sections showing the floral parts were retained and mounted.

After the middle of September the hairs on the inner side of each bud scale became so numerous it was impossible to cut the pear buds embedded in paraffin. It therefore became necessary to employ "Gibson's rapid process" of the colloidin method as described by Lee (*11*). Drinkard (*3*) was able to cut all collections of the Kieffer pear in paraffin, but it is the writer's belief that the Bartlett has a greater abundance of hairs than the Kieffer. After the January 1 collection it was possible to continue the paraffin method, since the flower-buds had then become large enough to permit the removal of the hairs without serious injury to the floral parts.

RESULTS

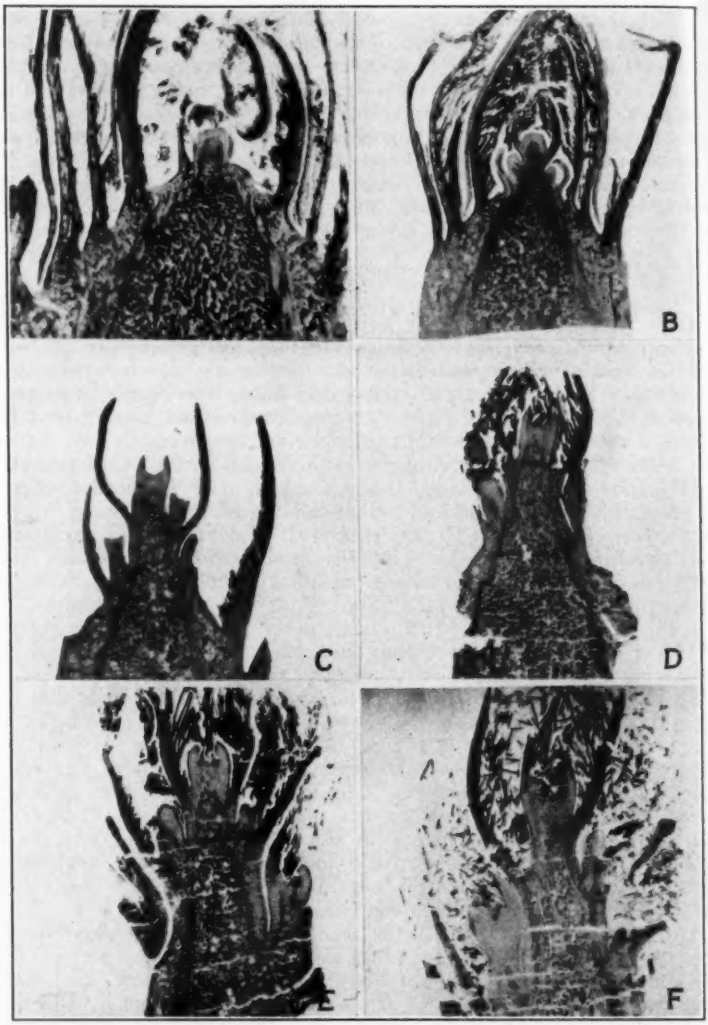
DIFFERENTIATION AND DEVELOPMENT OF FRUIT BUDS OF THE PEAR

Fruit buds were found among the buds collected July 7, 1922, from a lightly pruned and irrigated pear tree on the university farm (interior-valley conditions). However, since these flower buds were only in the first stage of differentation, it can safely be said that the Bartlett pear began fruit-bud formation the first week in July during the 1922 season. This finding is in accordance with that reported by Henderson.[4] At this time the growing point of the bud had ceased to give off bud scales and had become elongated and flattened on top. In the course of the next 10 days this crown had lengthened materially, and part of the specimens in longitudinal section showed the large terminal and two smaller lateral primordial flowers in the form of blunt protuberances (pl. 1). Each infant flower was subtended by a bract or modified leaf. It might be noted that the vascular system of the spur advances very closely with the elongation of the growing points and is lost sight of only near the tips.

In the collection made July 31 many buds were cut in which the terminal primordium had developed into a cuplike receptacle by a growth of tissue from its edges. The side primordia did not show as great development, but some had small projections at the edge. The August 10 samples showed little advance, except that in a few cases small round swellings on the inner surface of the receptacle cup showed the start of petals in the terminal flower. The entire bud slowly enlarged during the following 20 days, the primordial petals of the terminal flower became more prominent, and their beginnings became evident in the laterals.

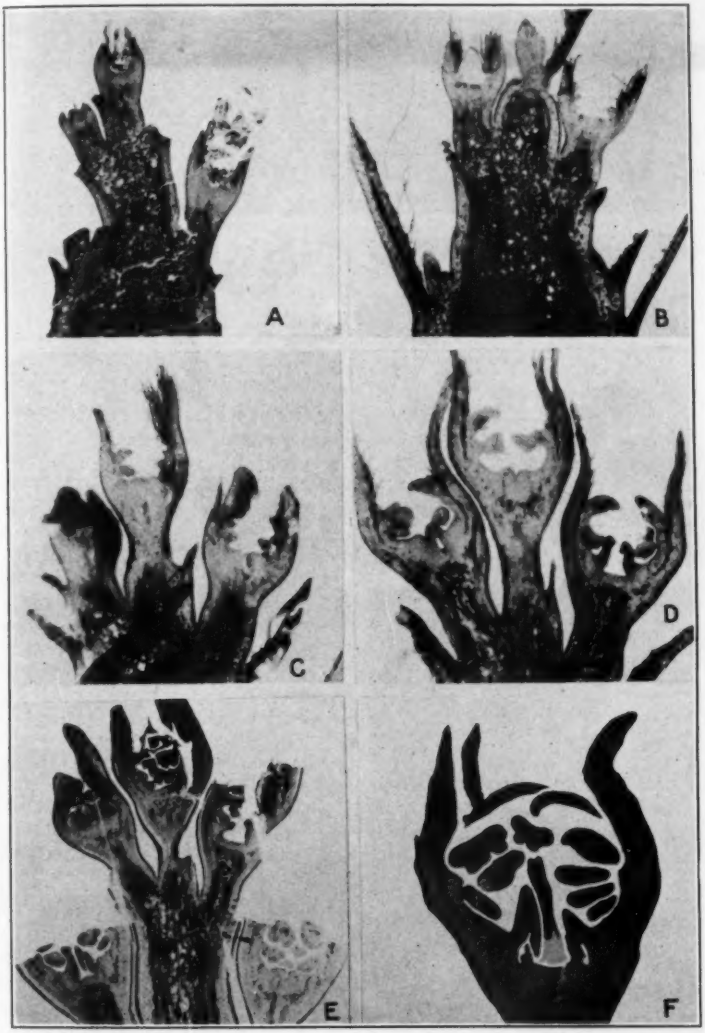
During September (pl. 2) the buds continued to enlarge slowly and the first whorl of stamens had their origin from the torus lining the inner side of the receptacle cup, immediately below the petals, and resembling the petals at the early stages. In nearly all cases the terminal primordial bud which was the oldest was the largest in size and farthest advanced. By October 30 the petals had lengthened and at least another whorl of stamens had made its appearance. It was not determined at what stage this whorl could first be seen, although there appeared to be much the same arrangement

[4] HENDERSON, W. THE DIFFERENTIATION AND EARLY DEVELOPMENT OF THE FLOWER BUDS OF THE BARTLETT PEAR. 1916. [Unpublished thesis, Univ. Calif.]



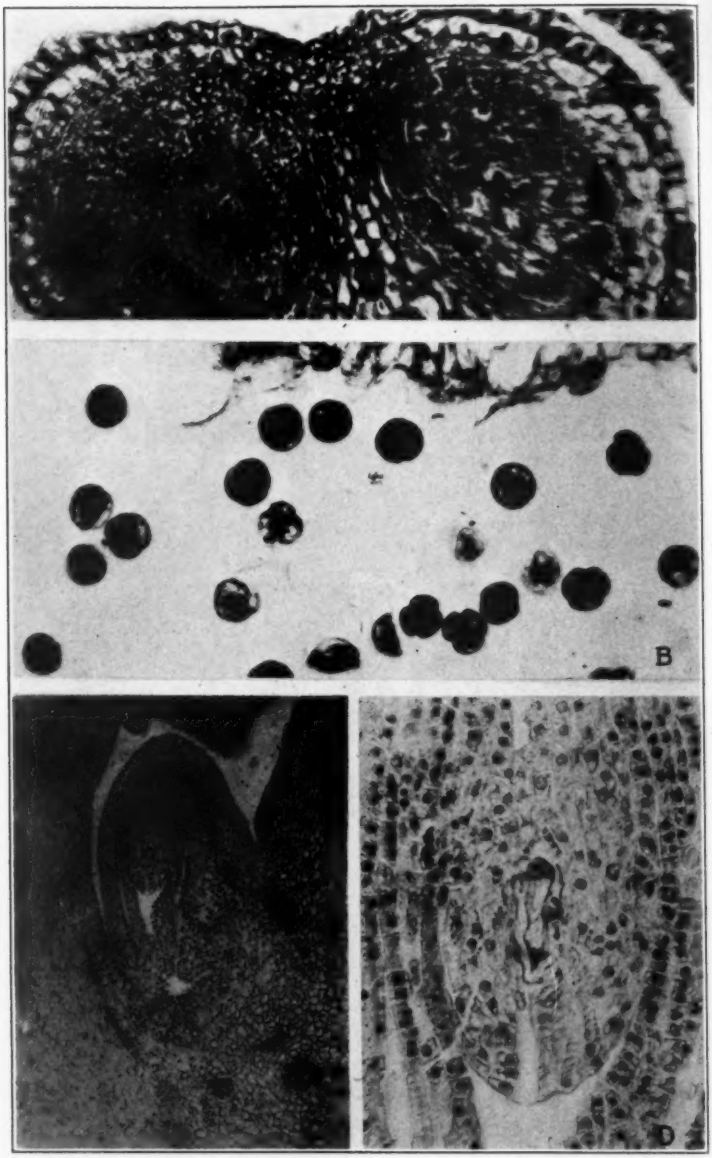
Photomicrographs of longitudinal sections through Bartlett-pear fruit buds collected from university farm orchard, showing the stage of development throughout the season of 1922–1923. (Approximately × 20)

A, July 7; B, July 21; C, July 31; D, August 10; E, August 21; F, September 2



Photomicrographs of longitudinal sections through Bartlett-pear fruit buds collected from university farm orchard, showing the stage of development throughout the season of 1922–1923. (Approximately × 20)

A, September 16; B, September 29; C, October 30; D, January 1; E, February 6; F, February 28



A.—Photomicrographs of cross-section through Bartlett-pear anther, February 21, 1923, showing the pollen mother cells. (× approximately 450)

B.—Photomicrographs of cross-section through Bartlett-pear anther, March 12, 1923, showing mature pollen grains. (× approximately 404)

C.—Photomicrographs of longitudinal section through the ovary of the pear, showing developing embryo sac. The nucellus is seen as an oval-shaped structure at the center surrounded by the inner and outer integuments. The large central cell of the nucellus is the embryo sac. (× approximately 125)

D.—Same as C, enlarged. This evidently represents an early stage in the development, as only one nucleus is found. (× approximately 355)

in later stages of the pear as Kraus (*10*) described for the apple. Small swellings on the bottom of the receptacle cup indicated the beginnings of carpels. Buds collected in December showed petals differentiated further and almost inclosing the stamens and carpels below. The stamens showed some differentiation into anthers and filaments, and styles were projecting upward from the carpels. In some cases cavities could be seen in the carpels.

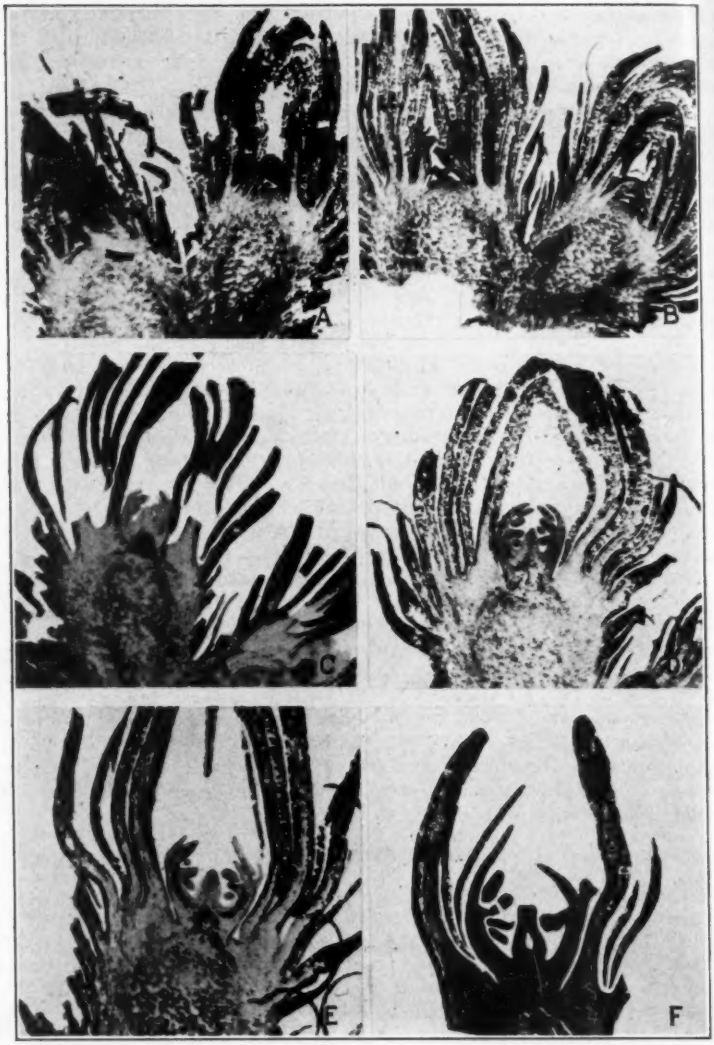
Mother cells (pl. 3) were clearly seen in the anthers of the buds gathered January 1. The mother cells were somewhat larger than the surrounding cells. They were located in the centers of the four lobes of the anthers and contained very large nuclei, staining dark red with safranin. The buds made little advance, except in size of anther, until about February 28. The mother cells had enlarged and become rounded, surrounding cells had differentiated into anther walls, and by the expansion of the anthers the mother cells had become more or less loosely associated. The five carpels had pushed up through the center of the bud into a well-defined pistil and two ovules could be seen as small knobs protruding from the enfolding placenta of the ovary. The petals completely covered over the other parts of the flower and appeared lanceolate in longitudinal section. Growth expansion was taking place in all parts and swelling of the buds could be observed by external examination in the field.

Well-defined tetrads, or clusters of the four granddaughter cells, were in evidence in material gathered March 8. This four-cell stage must be of short duration, because six days later each unit of the tetrad had broken away and was differentiated into a nearly, if not entirely, mature pollen grain.

On March 6 the stage of development of the anthers was much in advance of that of the carpels. The pollen grains were matured, whereas the ovules were at very early stages. One might be led to believe from this that the condition would result in a clear case of protandry. On the contrary, development in all parts of the pistil progressed from this date at an exceedingly rapid rate. From the small protuberances marking the start of the ovules on February 28, outer and inner integuments were well defined and inclosed the embryo sacs. Development in the remainder of the pistil was as rapid. The styles elongated greatly, and stigmas were differentiated, while enlargements of all parts took place.

DIFFERENTIATION AND DEVELOPMENT OF FRUIT BUDS OF THE APRICOT

The first collection of heavily pruned and irrigated Royal apricots on the university farm (interior-valley conditions) was made July 7. When these buds were sectioned it was found that the growing point was perfectly smooth, rounded slightly, and giving rise to bud scales from all sides. In this condition it was impossible to distinguish between future leaf buds and fruit buds. The same was true of all collections until July 31. At this date no definite signs of the initial fruit-bud stage were found, but the crown was giving rise to no more bud scales. On August 10 (pl. 4) the crown or growing point had become slightly elevated and flattened on top, while very small protuberances were observed at each side in longitudinal section, showing definite differentiation into fruiting buds. The sepal primordia were



Photomicrographs of the longitudinal sections through Royal-apricot fruit buds collected from university farm orchard, showing stages of development throughout the season of 1922–1923. (× approximately 23)

A, August 10; B, August 21; C, September 16; D, September 29; E, October 13; F, October 30

the first to appear. Walker [5] found differentiation started August 4 (1916) under similar conditions. It is interesting to note that the buds of a given date and condition that were observed were of surprisingly the same stage of development. As pointed out by Henderson,[6] pear buds of a given collection varied widely in the stage of development.

Little advance could be seen in the material collected August 21, as compared with that of August 10, except that the small protuberances or sepal primordia were more pronounced. However, by September 2 considerable advancement had taken place. The sepals had pushed upward and inward at the top, forming a cup, while the whole bud had broadened, except at the base. Primordia of petals were present inside the cup. They arose from the inner surface of the cup or receptacle. Also, the pistil was prominent as a blunt swelling from the floor of the cut. By September 16 all parts had further enlarged, and in addition to the sepals and petals the outer whorl of stamens was present. By September 29 the second and third whorls of stamens were present and all were of apparently the same stage of development. At the same date, owing to this elongation, the petals were drawing together above the stamens.

All parts continued to enlarge during October. By October 30 the petals, narrowly attached at the base, appeared lanceolate in section. Stamens were well differentiated into filament and anther, while in the four lobes of the latter pollen mother cells were observed. The pistil had elongated and the style could be distinguished from the broadening ovary. The ovarian cavity was present, but as yet no ovules were evident. By December (pl. 5) the petals had completely inclosed the bud at the top and the buds were slightly larger.

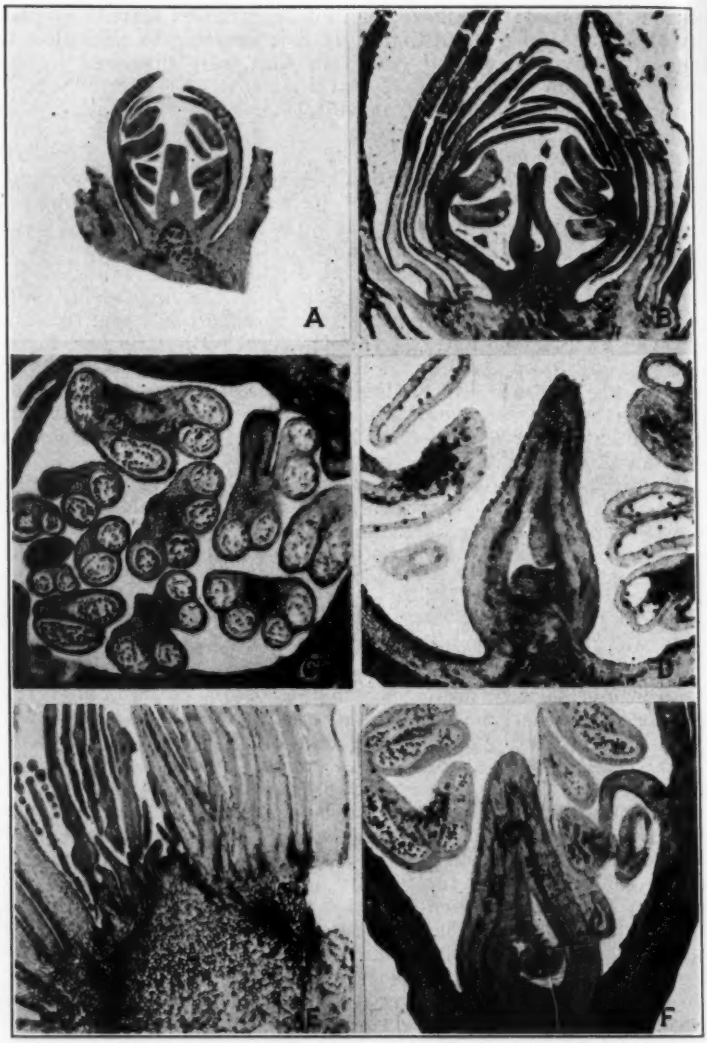
Buds collected January 1 showed a big increase in size of all parts. Ovule primordia were present as small knobs in the ovarian cavity, and the stigma had developed at the upper end of the lengthened style. A cross section of the ovary shows it not to be a perfect circle, but the walls fold in on one side, forming the future suture of the mature fruit. The ovules develop from the enfolded ends.

During January the buds continued to enlarge. The development in the ovary was slow. The stamens became larger and by February 6 had definite walls inclosing the large, rounded, loosely connected mother cells now ready for cell division (pl. 6). Material collected February 14 contained the four-celled or tetrad stage in pollen development. In apricots, as in pears, only one date showed this stage. By February 21 pollen was mature, but development of the ovule had progressed very little, as can be seen from Plate 5, D.

Following this stage, however, the development in the ovule progressed rapidly, and only five days later the two integuments and the nucellus were plainly evident in longitudinal sections of the ovary (pl. 5, F). The buds rapidly expanded, and the tree was in full bloom by March 2.

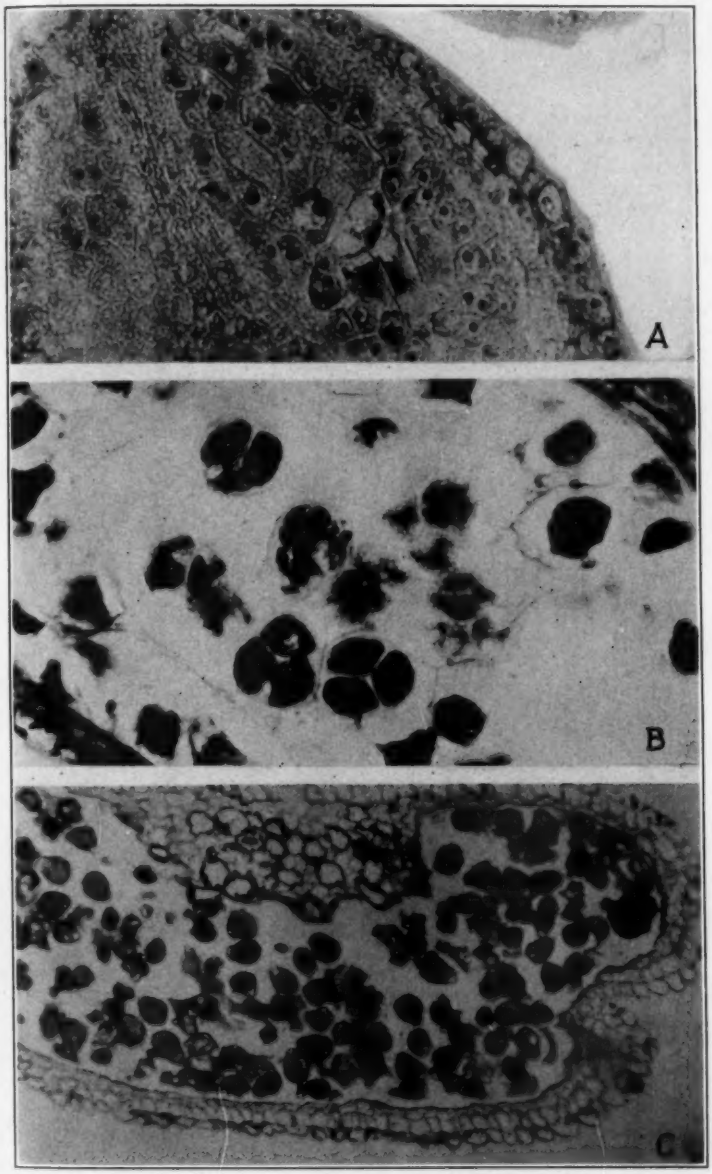
[5] WALKER, R. M. THE FORMATION AND DEVELOPMENT OF THE FRUIT BUDS OF THE ROYAL APRICOT. 1917. [Unpublished thesis, Univ. Calif.]

[6] HENDERSON, W. THE DIFFERENTIATION AND EARLY DEVELOPMENT OF THE FLOWER BUDS OF THE BARTLETT PEAR. 1916. [Unpublished thesis, Univ. Calif.]



Photomicrographs of the longitudinal sections through Royal-apricot fruit buds collected from university farm orchard, showing stages of development throughout the season of 1922–1923. (× approximately 25 unless otherwise stated)

A, December 1, 1922; B, January 1, 1923; C, February 6 (cross-section through anther, showing pollen mother-cell stage); D, February 21 (ovule development in a cross-section of the ovary); E, February 25 (longitudinal section through leaf bud, × approximately 30); F, February 26 (longitudinal section through ovary, showing further development of the ovule)



A.—Cross-section through a Royal-apricot anther, January 1, 1923, showing the pollen mother-cell stage. Note the large nuclei in the mother cells. (× approximately 371)

B.—Cross-section through a Royal-apricot anther, February 14, 1923, showing the tetrad stage in the development of pollen. Each member of the tetrad ultimately becomes a pollen grain. (× approximately 557)

C.—Mature pollen grains in cross-section of a Royal-apricot anther, February 21, 1923. (× approximately 138)

DIFFERENTIATION AND DEVELOPMENT OF FRUIT BUDS OF THE PEAR GROWN UNDER THE VARIOUS CONDITIONS

Heavy and Light Pruning Compared

Up to July 20 buds from the heavily pruned and irrigated pear tree were slightly more developed than those from the lightly pruned and irrigated pear trees at the university farm (fig. 2). After that, however, the slight differences that existed fluctuated from one to the other, so that on the average they were uniform in development from August 1. As previously stated, these two trees did not show conditions as extreme as would have been desirable. The lightly pruned tree blossomed about two days earlier than the other. The blossoming of lightly pruned trees somewhat sooner than heavily pruned ones has been noticed in previous years also.

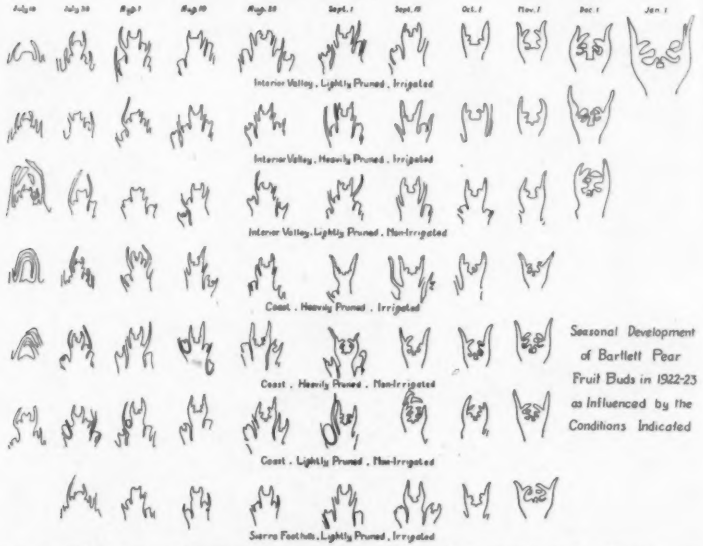

Interior Valley, Lightly Pruned, Irrigated
Interior Valley, Heavily Pruned, Irrigated
Interior Valley, Lightly Pruned, Non-Irrigated
Coast, Heavily Pruned, Irrigated
Coast, Heavily Pruned, Non-Irrigated
Coast, Lightly Pruned, Non-Irrigated
Sierra Foothill, Lightly Pruned, Irrigated
Seasonal Development of Bartlett Pear Fruit Buds in 1922-23 as Influenced by the Conditions Indicated

FIG. 2.—Outline drawings of longitudinal sections through Bartlett-pear fruit buds, showing the average stages of development on the various dates and under the various conditions

Although lightly and heavily pruned nonirrigated trees were observed in Sonoma County, the two trees did not grow near each other or on similar soil. However, when the comparison (fig. 2) was made between the two trees, it was found that the lightly pruned tree as compared with the heavily pruned one was in a more advanced stage of development when first examined (July 11) and kept slightly in the lead until August 21. At this date and afterwards the development was approximately the same under the two conditions.

Irrigation and Nonirrigation Compared

On the university farm the fruit buds from the nonirrigated pear tree were farther advanced than those from the irrigated tree on July 7 and 20 (fig. 2). After the latter date, however, slightly the reverse

was the case. Both the heavily and lightly pruned and irrigated trees were slightly ahead of the nonirrigated tree on November 1, but there was no apparent difference December 1.

In Sonoma County the buds from the nonirrigated tree seemed to have a lead over those from the irrigated one throughout the season; both trees were heavily pruned (fig. 2). The lead was slight on July 11, but by September the buds from the nonirrigated tree were about 30 days in advance. These buds were noticeably ahead when the last samples were taken. Buds grown with the combined influence of nonirrigation and light pruning were slightly farther ahead of the heavily pruned and irrigated than of the heavily pruned and nonirrigated.

Regions Compared

Comparison of pear buds taken from Eldorado (Sierra-foothill conditions) and Yolo Counties (interior-valley conditions) under the same cultural treatment (fig. 2) showed that the buds grown under foothill conditions were, on July 20, slightly behind those from the interior valley and developed at a slower rate until the middle of August. Later collections, however, showed a rapid development in the buds from Eldorado County, which were leading October 1. By November these buds had reached a stage of development that buds grown under interior-valley conditions required from two to four additional weeks to reach.

Although pear fruit buds in the coastal valley and in the foothills apparently differentiated at about the same date, those in the cooler and more humid section developed more rapidly than those at the high elevation. The buds from the coastal valley were at least 30 days ahead by the middle of August. However, the buds grown under foothill conditions made such advance during September that the stages of development were equal October 1 and the Eldorado buds even slightly in the lead by November 1.

DIFFERENTIATION AND DEVELOPMENT OF FRUIT BUDS OF THE APRICOT GROWN UNDER THE VARIOUS CONDITIONS

Light and Heavy Pruning Compared

Pruning and irrigation treatments of apricot trees could not be compared in respect to fruit-bud formation, except at the university farm. The lightly and heavily pruned trees were irrigated. Both showed the initial signs of fruit-bud formation about August 10, and buds from each developed equally until the middle of September (fig. 3). Collections made September 15 and October 1 indicated the heavily pruned tree to be considerably ahead in floral development. This lead was not maintained, however, and the lightly pruned tree had buds showing the ovule primordia December 1, while none were found in buds taken from the heavily pruned trees.

Irrigation and Nonirrigation Compared

Fruit buds taken September 15 from the nonirrigated apricot tree in the university orchard showed a slight lead over those from irrigated trees, and they maintained it as long as collections were made (fig. 3). Prior to September 15, no difference in degree of development was observed.

Regions Compared

Buds taken August 1 from the tree in the coastal-valley section had smooth crowns, while no signs of this condition were evident in either of the other locations. However, in the subsequent collections the coastal-valley apricots seemed to be slightly behind, especially in pistil development, until the collections of October 15 (fig. 3). Buds collected from the coastal-valley district at this latter date showed a stage of development which buds from the other two locations required an additional month to reach. These more advanced buds contained well-defined pollen mother cells on October 15.

The apricots grown in the foothills and in the interior valley showed differentiation of fruit buds on August 10, which developed at about the same rate until in September, when the buds grown at the higher

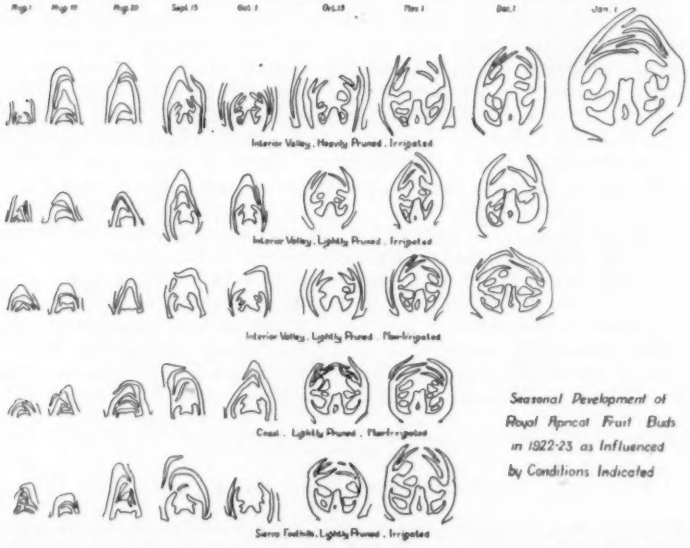

Interior Valley, Heavily Pruned, Irrigated
Interior Valley, Lightly Pruned, Irrigated
Interior Valley, Lightly Pruned, Non-Irrigated
Coast, Lightly Pruned, Non-Irrigated
Sierra Foothills, Lightly Pruned, Irrigated
Seasonal Development of Royal Apricot Fruit Buds in 1922-23 as Influenced by Conditions Indicated

FIG. 3.—Outline drawings of longitudinal sections through Royal-apricot fruit buds, showing the average stages of development at the various dates and under the various conditions

altitude assumed a slight lead over the buds grown in the valley. At this time the buds under foothill conditions had anthers in the mother-cell stage of development.

DISCUSSION

With the long growing season in California it would seem logical to suppose that fruit-bud differentiation would take place earlier than in the East. This assumption appears well founded, since the Kieffer in Virginia (*3*) and the Wilder Early in Wisconsin (*6*) were found to show signs of fruit-bud differentiation about three weeks later than the Bartlett in California. Disregarding varietal differences, the late differentiation of pear fruit buds in Virginia and Wisconsin, as compared with the early fruit-bud formation in Cali-

fornia, might be explained by the relatively early beginning of the growing season in the latter State. Whether this or something else is the right explanation, it is clear that there are differences between localities. The writer knows of no data on the time of fruit-bud differentiation of the apricot except in California.

The development of fruit buds under interior-valley conditions is characterized by a slow but steady development of the floral parts through the summer and winter, with a rapid growth, of ovules especially, in the spring just before blossoming. Winter seems not to have any appreciable checking influence, although buds in California, even though blossoming earlier, are at a less advanced stage in December than are buds at the same time in the Eastern States.

Apricot buds differentiate a month or more later than pear buds, but develop at a much more rapid rate, and come into blossom earlier.

It would seem from the data collected that the differentiation both of the pear and apricot fruit buds, respectively, takes place at approximately the same time in all regions from which samples were taken. It is interesting, however, to note the more rapid development under coastal-valley influences. Development of fruit buds from the foothill sections, though slow at first, was only slightly less rapid than under coastal-valley conditions, while the stages of development of buds collected under interior-valley conditions were the least advanced November 1. In the case of the pear fruit buds, the lead of those from near the coast was maintained from the start, and this lead increased steadily until in October when they were about a month ahead of those grown at the higher altitudes. Development of fruit buds under foothill conditions was almost at a standstill following differentiation until September, when it progressed rapidly, due perhaps to the cooler weather there.

Gardner, Bradford, and Hooker (*5*) state that the rather restricted period of fruit-bud formation can be varied somewhat by cultural treatment, including, perhaps, any practice that modifies the rate of growth. As summer pruning has an influence on the time of fruit-bud differentiation (*4*), and since light and heavy pruning, popularly known as "long" and "short" pruning, cause very different reactions in other respects, why, then, should not light and heavy dormant pruning operate differently in respect to fruit-bud differentiation? Since heavy pruning induces much shoot growth, one might expect it to cause growth later in the season and thereby bring about later differentiation. This was found to be rather strikingly the case with the two pear trees under coastal influences. These trees grew on two types of soils, which may have caused the difference. When pruning methods were compared in the interior valley, the heavily pruned pear tree differentiated fruit buds a few days in advance, the reverse of the observation at the other location; however, the lightly pruned tree developed somewhat faster through July and August. With the exception of the collections made September 15 and October 1, the heavily and lightly pruned apricot trees on the university farm showed the same rate of development. It would seem from the data available, at least in the case of the pear, that light pruning as practiced in California tends to hasten development.

Contrary to Goff's findings (*7*), and coinciding well with Kirby's conclusions (*9*), the lack of moisture apparently tends to hasten fruit-bud differentiation and subsequent development. The retarding

influence of irrigation on fruit-bud development of the pear was most strikingly shown under coastal-valley conditions. Only buds collected July 7 and 21 from nonirrigated trees on the university farm showed any lead over those taken from irrigated trees. No difference in development of fruit buds of irrigated and nonirrigated apricot trees under interior-valley conditions could be observed until September 15. At this date the buds from the nonirrigated trees assumed a lead which they maintained as long as collections were made.

SUMMARY AND CONCLUSIONS

A study was made of the influence of certain cultural and environmental factors on the differentiation and development of Bartlett pear and Royal apricot fruit buds. In order to study the environmental influences upon fruit-bud formation and development, material was collected at short intervals between July 7 and November 1, 1922, and from three typical fruit-growing regions of California, namely, Sierra foothills (Eldorado County), interior valley (Yolo County), and coastal valley (Sonoma County). In addition, samples under interior-valley conditions were collected until blossoming time. To study the effect certain cultural practices might have, lightly pruned trees were compared with heavily pruned ones, and irrigated were compared with nonirrigated trees in respect to time of fruit-bud formation and the rate of development. The following conclusions seem justified:

1. Pear fruit buds begin to differentiate at approximately the same date under coastal-valley, interior-valley, and foothill conditions.

2. Apricot fruit buds begin to differentiate at approximately the same date under coastal-valley, interior-valley, and foothill conditions.

3. The high altitude of the foothills seems to have a retarding influence on fruit-bud development until the middle of September when development becomes more rapid.

4. The humid coastal conditions apparently stimulate rapid development of pear buds after differentiation. This is not the case with apricots until October when development becomes extremely rapid and the buds go into the winter at a more advanced stage than is found under either interior-valley or foothill conditions.

5. The dry, hot, interior valley seems to induce a steady uniform development of both pear and apricot fruit buds; however, these have not reached the advanced stage of development by early winter that buds from the coastal valley and foothills have attained.

6. The inception of fruit-bud differentiation seemingly is not influenced to any extent by either heavy or light dormant pruning. Light pruning perhaps tends to induce a slightly more rapid development for six to eight weeks following fruit-bud differentiation of the pear.

7. Irrigation seems to have a retarding influence on fruit-bud differentiation and development.

8. Environmental conditions during winter, as found in the principal fruit-growing districts of California, do not seem to have any checking influence on fruit-bud development of the pear and apricot.

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BENZENE AS A LARVICIDE FOR SCREW WORMS[1]

By D. C. PARMAN

Assistant Entomologist, Investigations of Insects Affecting the Health of Animals, Bureau of Entomology, United States Department of Agriculture

INTRODUCTION

The larval stage of *Cochliomyia macellaria* Fab., generally known among stock raisers in the Southwest as the screw worm, causes considerable loss to the livestock industry, estimated as high as $5,000,000 in some years. It has been apparent that the larvicides used to kill the worms are either toxic to the animal or at least in most cases detrimental to the healing of the wounds. This toxicity was at first attributed to the screw worm, but as many cases were observed where the animal was practically consumed by the larvae and still lived until the loss of blood or injury to some vital organ brought death, it was surmised that the treatments with larvicides were the cause of many deaths. During the summer of 1916 systematic work was begun to find a more efficient larvicide than the phenols and chloroform which were generally used.

At first an attempt was made to add something to these larvicides to counteract the toxic properties. As this was not successful it was deemed best to look for a chemical that might be used with more satisfactory results. Several chemical groups were studied for possible larvicides.

EXPERIMENTAL PROCEDURE

All available chemicals with possible larvicidal value were selected for laboratory tests to determine whether they would kill the larvae of the screw-worm fly. The first tests were made by pouring the chemical on a number of larvae in a tube, or dusting on just enough to cover the larvae. All chemicals that stood this test were selected for tests on infested rabbit carcasses; and from these, after the elimination of the ones deemed too toxic at the greatest dilutions to be used on wounds, the others were selected for field tests on living animals.

CHEMICALS USED IN FIELD TESTS

Ethyl ether, benzene, toluene, xylene, cymene, carbolic acid, safrol, eugenol, ortho-, meta-, and para-cresol, nicotine, pyridine, nitrobenzene, methyl chloride, chloroform, carbon tetrachloride, methyl alcohol, amyl alcohol, formaldehyde, picric acid, formic acid, carbon disulphide, sodium cyanide, mercurous chloride, mercuric chloride, borax, sodium arsenate, antimony chloride, benzine, gasoline, kero-

[1] Received for publication February 21, 1925; issued January, 1925.

sene, fuel oil, crude petroleum, coal-tar dips, creosotes, and turpentines were used in field tests.

These substances were first tested in very minute quantities on laboratory animals, many being diluted with light paraffine oil or water. All that proved markedly toxic or irritating to the animal as the amounts used increased to a point that would kill the screw worms were eliminated. The remaining ones were then tested, at first on small wounds, and, when no marked toxicity or irritation was observed, they were used on still larger wounds. The elimination process continued until only ethyl ether, benzene, toluene, xylene, chloroform, and carbon tetrachloride were used.

Ethyl ether was considered too volatile for field use, and was eliminated early for this reason. Toluene and xylene were about as effective as benzene but were eliminated because they were less available than benzene. Chloroform, chemically pure (not decomposed), was found to be quite satisfactory. Samples other than the chemically pure product, however, were found to be objectionable for the same reasons as held for the decomposed chloroform. Carbon tetrachloride and the chemically pure chloroform have a laxative effect on the wound that is undesirable.

BENZENE AS A LARVICIDE

Benzene has the same rank as a larvicide for the screw worm as ethyl ether, chloroform, carbon tetrachloride, and xylene; the larvae in each case, when moistened with these materials, became inactive in an average of 40 seconds. In dry wounds, the time required to kill the larvae is practically the same for each and is about the same as that for the larvae moistened with the chemicals. Where the wounds do not have good drainage, the larvae are killed very slowly with benzene, ethyl ether, and xylene, as these materials float on the blood and serum, and it has been found that the larvae can live submerged in the wound from 20 to 30 minutes. Chloroform and carbon tetrachloride sink into the blood and serum and mix with it, and all larvae are killed nearly as quickly as in a dry wound. To kill the larvae quickly with benzene in such wounds it is necessary to dry the wound first with cotton or a cloth and to apply the benzene immediately.

EFFECT OF BENZENE ON WOUND

Most wounds infested with screw worms have a considerable flow of fresh blood and serum. As long as the wound is dry it is not so attractive to the flies, and for this reason any chemical that has a tendency to laxate the wound is not desirable. Benzene acts as a styptic, and the blood flow stops in most cases as soon as all of the larvae are killed. When benzene is applied, and the wound takes on a dry, pale appearance, which lasts from 5 to 10 minutes or more, a wound dressing and fly repellent which adheres well to all parts should be applied, making a perfect coating which should continue the styptic effect. In no case has any indication of injury to the tissues been observed after an application of benzene, and the wound usually heals soft and dry without scabbing or cracking. On clean, smooth wounds there is hardly ever any dead tissue after treatment.

TOXICITY OF BENZENE TO ANIMALS

No tests have been made to determine the toxicity of benzene to the higher animals except in its practical use as a larvicide for the screw worm. No toxic effect has been observed on sheep and goats where the wool and mohair were entirely saturated on all parts of the body, and wounds as large as 8 inches in diameter were treated with sufficient amounts to kill all larvae in them. Such animals have in all cases been healed when they were kept under observation, although in some instances they were down and unable to move when treatment was begun. Goats, sheep, and calves quite commonly have infestations of worms in the mouth to the extent that all of the teeth are lost. In treating these cases care has been used to prevent the animals from swallowing any of the benzene and it is applied in small amounts (2 to 5 c. c.). In no case has any ill effect on the animals been observed.

In the treatment of more than 3,000 cases of worms in cattle, sheep, goats, hogs, dogs, and chickens, there has never been any noticeable toxic effect on the animal. In the work on the range it has been remarkable and significant to note the number of animals reported "as good as dead" by the herders that recovered completely after treatment. Most ranchmen have had the idea that any serious infestation with screw worms would kill the animal and for this reason many animals badly infested with worms have been killed and burned. In 2,843 cases of infested animals treated with benzene under the writer's personal observation, only 5 have died. In 3 of these cases the worms had entered the body cavity or were present in the head of the animal and had affected the nervous system.

Holland [2] makes the following statement: "A narcotic effect is produced by accidental inhalation of benzene vapor in factories. One ounce (30 c. c.) taken by the stomach caused death after symptoms such as headache, giddiness, bluish flush of the face, delirium, convulsions, and coma." While a wound in a big steer was being treated a pint bottle was kicked by the steer and broken and the contents thrown into the writer's face. There was some difficulty in breathing for about two minutes. The excess was wiped away and the face and eyes had a numb, cool feeling for about five minutes; there was then a slight burning sensation in the eyes which lasted about ten or fifteen minutes, succeeded by a very pleasant, clean, sensation in the eyes. No other symptoms were noticed at the time or later. When benzene is spilled on clothing and penetrates to the skin there is a burning sensation, but to the writer's knowledge no injury has ever been observed.

BENZENE IN PRACTICAL USE

Besides the experimental work in which benzene has been used, approximately 5,000 animals or more have been treated by ranchmen and herdsmen. The benzene was furnished by the Bureau of Entomology and reports were made to the bureau. Fifty-three ranchmen have been furnished benzene in amounts of 1 pint to 1 gallon each. Reports have been received from all of these and some were quite

[2] HOLLAND, J. W. A TEXT-BOOK OF MEDICAL CHEMISTRY AND TOXICOLOGY. Ed. 5, rev., 683 p., illus. Philadelphia and London. 1917.

complete as to the efficiency of the material as a larvicide, its effect on wounds, and toxicity to animals. In no case has any indication of toxicity to the animals or ill effects on the wounds been reported. In a few instances the reports were to the effect that benzene was slow to kill the worms in some cases. These probably were cases in which the wounds were not well drained and were not cleaned before benzene was applied. Most of the men feel that it is a very efficient larvicide and, on account of the effect on wound and animal, prefer it to any other they have ever used.

AVAILABILITY AND PRICE

Benzene used in the work has been of the grades known in the trade as "commercially pure" and "90 per cent." These grades are available to the trade in amounts that will be far beyond the demand for larvicidal purposes, and the source is practically inexhaustible. Commercially pure benzene is usually about 2 cents per gallon cheaper than the 90-per cent in quantity lots. Quotations on the commercially pure benzene, for June, 1924, in tank lots, were as low as 21.5 cents per gallon; in drums, 28 cents per gallon; these prices at production centers.

CONCLUSIONS

Benzene is an effective larvicide for the screw worm, when used properly. It has no ill effects on the wounds, and acts as a styptic temporarily to make the wound less attractive to the adult flies. When used in wounds as a larvicide for the screw worm it has not been found to be toxic to any animal.

METHODS OF STIMULATING GERMINATION OF WESTERN WHITE-PINE SEED[1]

By J. A. LARSEN

Forest Examiner, Priest River Forest Experiment Station, Forest Service, United States Department of Agriculture

INTRODUCTION

In forestry as in agriculture seeds are often encountered which are difficult to germinate and are called "rebellious." In this class belong the seeds of many hardwood species and not a few conifers. The soft or white pines are much noted for this characteristic, *Pinus monticola* in particular.

Prompt germination of western white-pine seed has constituted a real problem in the Forest Service nurseries of the northern Rocky Mountain region. In one instance 35 beds spring-sown with 5 pounds of seed per bed gave a meager and irregular germination the first summer and from 3,000 to 40,000 seedlings per bed the second season. Such results add greatly to the expense of raising nursery stock, in that the beds require care for two years instead of one and occupy double the usual space.

When this problem was taken up by the field station of the Priest River Forest Experiment Station, in northern Idaho, it was assumed as a working basis that delayed germination was due to inherent physiological characteristics requiring after-ripening of the seed or a period of rest,[2] or to the impermeability of the seed coat to water from without and to the root tip from within. If the first hypothesis were true, some process of stimulation by stratification or by chemical or physical means might be effective. If the latter were the case, reduction of the seed coat by chemical or mechanical means would be likely to bring success.

German investigators, among them E. Zederbauer,[3] who has done considerable work with conifer seeds, maintain that there is a relation between the time of ripening and of germination, and that the seed which is shed in the autumn germinates slowly while that which is shed in the spring germinates more rapidly. Zederbauer places *Abies alba*, *Pinus cembra*, and *Pinus strobus* in the slow class. The seed of these fall in the autumn. *Pinus sylvestris*, *Pinus nigra*, *Picea excelsa*, and *Larix europea* he places in the rapidly germinating class. The seed of this latter class is shed in March, or, as with *Picea excelsa*, in February. The seed which remains on the ground during the winter is subjected to the influences of water and frost, differences in ground conditions and other factors, all of which may produce chemico-physiological changes.

Early work on this project at the Priest River Forest Experiment Station embraced treatment with sulphuric acid and other chemicals.

[1] Received for publication Dec. 22, 1924; issued January, 1925.

[2] COULTER, J. M., BARNES, C. R., and COWLES, H. C. A TEXTBOOK OF BOTANY FOR COLLEGES AND UNIVERSITIES. v. 2, p. 931–932, illus. [1911.]

[3] ZEDERBAUER, E. DIE KEIMPRÜFUNGSDAUER EINIGER KONIFEREN. Centbl. Gesam. Forstw. 32: 306–315, 1906.

Tests were also made in different media and germinators, but with poor results. The invariably low germination obtained in all of the tests, even over long periods up to 200 days, led to the belief that the quality of the seed itself might be impaired either by storage or extraction. As soon, therefore, as new seed could be collected it was extracted from the cones under low temperatures and stored in air-tight containers. A complete series of new experiments, covering more than 300 different tests, was then outlined. These embraced stratification in different media for various lengths of time, with or without heat or cold treatment, soaking for different periods of time in acids and various chemicals, heating and freezing the seed, abrasion of the seed coat by cutting or other mechanical treatment, and studies on the need of after-ripening and necessity for a period of rest.

STRATIFICATION

Stratification of the seed as a preliminary treatment was suggested at the outset, for in this manner the seed would receive a treatment in bulk under conditions similar to those in the seed bed but without sowing and carrying the bed for two seasons.

The seed was laid 5 inches deep in 10-inch wooden boxes, with tops and bottoms of wire mesh to protect from mice and admit heat, cold, moisture, and provide drainage. Sand, sawdust, clay loam, and duff from the forest floor were used in parallel boxes. One series of these was sunk to the upper level of the box in a sandy bench, another in clay loam, a third on moist bottom land under young timber, and a fourth in clay loam in a cellar. Repeated installations were made March 5, June 6, and September 5, in order to determine the effect of the length of time kept in this condition. Sowings from all of these were made in the nursery early and late in the spring and autumn of the same year, and in the spring of the following year.

At the time of the first sowing, certain samples were given from 3 to 30 days additional treatment by heating the seed in moist greenhouse sand and from 3 to 9 days in moist cotton flannel in the incubator. In sand the temperature records show a variation from 70° to 100° F. and in the incubator from 80° to 102°.

When the first lots were removed from the stratification boxes, 37 days after being laid down, from 3.7 to 6.3 per cent of the seed had germinated, the lowest percentage being obtained from that sown in the clay in the cellar, and the highest in the clay on moist bottom land. Decay had also set in, but in no case was more than 1 per cent of the seeds affected, and the greatest amount of decay occurred with the highest germination. The germination continued during the heating in the sand and in the incubator and in some cases attained 1 per cent in 9 days, and in one case 8.6 per cent in the greenhouse sand in 30 days.

These seeds and also those taken directly from the stratification boxes were sowed in the nursery April 20, together with a check sowing. Three beds were used, one of which was open, another covered with a sphagnum-moss mulch, and a third covered with tar and felt paper. Triplicate samples representing each individual treatment were thus sowed in parallel series, one in each of these beds.

The great number of tests and the many mediocre results make it futile to give the results in detail. Only the best results from each location and medium are given (Table I).

TABLE I.—*Maximum germination according to each location and medium for stratification*

Stratification		Secondary treatment		Germination [a]
Location	Medium	Nature	Period	
			Days	*Per cent*
Clay loam bench	Sandy	Greenhouse	9	[b]19.5
Do	do	Incubator	9	19.5
Do	Clay loam	do	9	23.0
Do	Sawdust	do	3	39.0
Do	Duff	do	9	21.0
Loamy sand	Sandy	do	3	32.0
Wet bottom land	Clay loam	Greenhouse	6	[c]39.0
Clay loam cellar	do	Incubator	9	14.5
Not stratified	Check	do	3	31.0
Do	do	Greenhouse	30	32.0
Do	do	No heating		21.0
Clay loam bench	Clay loam	Tips cut with knife		32.0
Not stratified		No heating; tips cut with knife		67.0

[a] Seed bed covered with moss mulch, except as otherwise noted.
[b] Seed bed uncovered.
[c] Seed bed covered with tar paper.

Though showing a slight increase in some cases over the check tests, this method of stratification failed to give satisfactory results. Thirty days' simple treatment in the warm moist sand in the greenhouse or three days' moist heating in the incubator gave almost as good returns as did any of the stratified samples.

Germination by autumn sowing of the earliest as well as the later stratified seed, and also germination from the spring sowing of all of these the following year, were much below the percentages in Table I, even below the earlier check tests. Failure was shown by careful cutting tests to be due to rotting of the seed.

Germination of seed placed in sawdust was slightly better in most cases than from the other media, and the later tests show that the seed may remain unimpaired longest in sawdust. Bark-free sawdust was used in order to avoid the possibly injurious effect of tannin.

The tests bring out very clearly the beneficial effect of a moss mulch in aiding germination, for in more than 90 per cent of the cases the highest tests occurred in the mulched bed; and this method of preserving moisture of both air and soil and of equalizing the temperature promises better germination in seed beds in Montana and Idaho, where the atmospheric humidity is lower and the diurnal fluctuations in temperature are greater than in the Central or Eastern States.

CHEMICAL TREATMENT

The treatments designed to hasten germination through corrosion of the seed coat by chemicals included soaking for periods of 8, 15, 25, 35, and 45 minutes in concentrated solutions of sulphuric, acetic, and citric acids. One of the samples was heated by the addition of water to the sulphuric acid. Some were treated for 15, 30, 60, 120, and 180 minutes in saturated potassium permanganate and potassium sulphate, and others for 5, 10, 20, 30, and 60 minutes in carbolic acid. Each of the samples chemically treated was washed in 12 waters to prevent further absorption and chemical injury to the seed kernel.

Samples of seed were frozen in an ice-cream freezer for 6, 12, 18, and 24 hours after having been soaked in cold water to test the effect of the mechanical action of freezing. One sample was soaked in full-strength sulphuric acid 15 minutes, thoroughly washed, and frozen for 24 hours; another was frozen 24 hours and then heated in the incubator for 3 days.

The results of these treatments and of the several check tests, as well as of samples with holes cut in the seed coat, are given in Table II:

TABLE II.—*Highest germinations obtained by soaking, freezing, and chemical treatments*

No.	Treatment	Time	Germination in seed bed (250 days)			
			Uncovered	Moss mulch	Check tests: Uncovered	Check tests: Moss mulch
			Per cent	*Per cent*	*Per cent*	*Per cent*
1	Sulphuric acid	45 minutes	38.5	28.5	4.5	3.5
2	Sulphuric acid, then heated to 144° by water and left standing 30 minutes.	do	40.5	30.0	6.0	5.5
3	Acetic acid	40 minutes	7.0	14.0	5.5	10.0
4	Citric acid	120 minutes	7.0	5.5	8.5	2.5
5	Sulphuric acid	15 minutes	11.0	26.5	7.0	10.0
	Freezer	24 hours				
	Incubator	3 days				
6	Potassium permanganate	180 minutes	19.5	19.5	7.5	7.5
7	Potassium sulphate	120 minutes	26.5	24.5	5.5	4.0
8	Carbolic acid	30 minutes	15.0	12.5	5.0	4.0
9	Tips worn by rubbing on file		34.0	21.0		
10	Tips cut with knife		51.0	56.0		
11	Seed coat cracked by crushing		0.0	1.0		
12	Seed coat split open with knife		41.0	29.0		
13	Hole cut in seed coat, leaf end		40.0	22.0		
14	Soaked in sulphuric acid until seed coat could be rubbed off.		24.0	42.0		
15	Seed coat entirely removed		43.0	4.0		

In the tests with chemicals the highest nursery germination of 38.5 per cent was obtained by soaking the seed in concentrated sulphuric acid for 45 minutes. The addition of water to produce heat raised this to only 40.5 per cent and is not considered a sufficient gain to be worth while. The treatments with the other chemicals resulted, on the whole, in very little stimulation. The soaking in acid 15 minutes with later freezing and heat treatment produced germination no better than soaking in sulphuric acid 35 minutes without freezing. Freezing as well as heating by these methods would therefore seem to be useless operations.

Not only a greater total germination but a more rapid rate than in the sulphuric acid tests was obtained by the mechanical treatments, especially the cutting of a very small hole in the end of the seed with a knife. (See fig. 1.) Mechanical abrasion of the seed coat, if it can be done without injury to the kernel, is therefore sure to give a higher and more rapid germination than either stratification or chemical corrosion of the seed coat.

Preliminary treatment to stimulate the germination of white pine by soaking in sulphuric acid is a practical and efficient method which

may be used on a large scale for spring sowing at the nursery. It requires only a zinc-lined vat as acid container, running water, and paraffined hardware cloth baskets for dipping and rinsing the seed. After the treatment the acid is somewhat colored but has not lost its value for sterilizing purposes. Dry seed only must be used for the acid treatment, for experiments have shown that wet or soaked seed are injured by the acid.

These tests, particularly those in which the seed coat was removed, show beyond doubt that delayed germination in the seed bed the first season after collection is due entirely to the obstruction of the seed coat itself and not to any inherent physiological character of

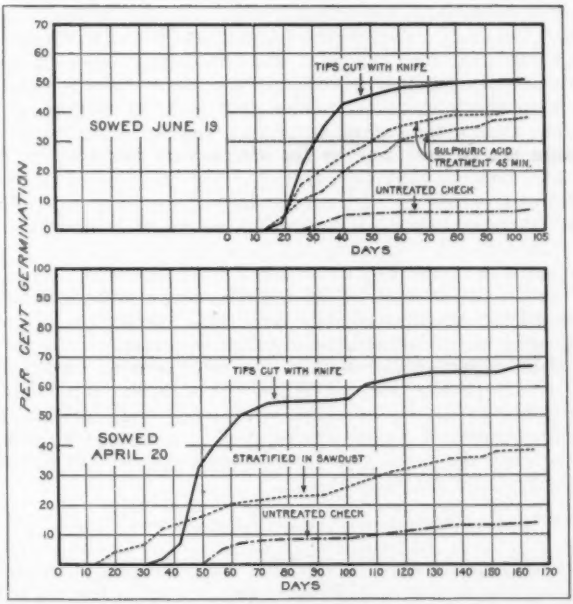

FIG. 1.—Nursery germination of western white pine

the seed. After-ripening or the need for a period of rest may still be factors to contend with, but at any rate these do not hinder germination during the spring and summer following seed production.

MECHANICAL TREATMENTS

On the strength of the excellent results obtained from the cutting or removal of the seed coat, experiments were undertaken to devise a churning apparatus coated with emery or carborundum dust for the reduction of the seed coat. At this time, however, T. R. Truax had produced such a machine by which red clover germination was materially hastened. Accordingly, several samples of western white-pine seed were sent for treatment.

Tests of the earliest samples by this treatment gave negative results, because most of the seeds were broken or crushed, but in the second tests 16.5 per cent germination was obtained in 40 days compared with only 11.3 per cent in the checks. Parallel samples with tips cut gave 20 per cent in the same period. But though germination was hastened by this means, the seed thus treated eventually gave a lower test than the untreated because of mechanical injury.

This shows that it may be entirely feasible to increase the rate of germination by scarification, provided suitable machinery is available. Though the perfection of such machinery is beyond the province of the present project, a modified type of barley pearling machine is suggested as suitable for this purpose. In this machine the grain is scarified between a perforated outside steel casting and an inside revolving carborundum wheel. For white-pine seed it would be necessary to substitute a fleece-lined sheepskin for the steel casting so as not to crush the seed. The process of scarifying white-pine seed, however, is more complicated than rice or barley, because very little pressure can be used to force the seed against the abrasing surface, and because the resin will clog the machine very quickly.

A series of treatments with coarse and fine needles was tried later, the chief purpose being to determine whether or not germination could be hastened by blowing the seed against a bank of needles. No machine was made, but the seeds were punctured by the needles as they lay on the blotter. The needles were prevented from penetrating deeper than through the seed coat by surrounding the tip with sealing wax. The bank of needles was made by setting them in a piece of paper similar to pins stuck in a folded strip of paper and rolled compactly and solidified by sealing wax. This bank made 32 points of needles within a circle one-fourth inch in diameter.

By this treatment the seed was injured, presumably by mechanical impact, for only one sample of those treated showed any germination, and this only 5 per cent in 40 days.

EXPOSURE TO LOW TEMPERATURES

Additional experiments in soaking and freezing the seed were made during the winter of 1916 in order to try the mechanical effect of the water in freezing and thawing and of exposure to low temperatures for a longer period in air and snow. This work was undertaken largely on the strength of the excellent results recorded by Pittauer [4] in stimulating germination of *Pinus strobus*. With dry freezing and 24 hours' soaking after the freezing, he obtained 75 per cent germination in 28 days, and with snow freezing he obtained 56 per cent, with only 17 and 18 per cent for the untreated samples.

The Priest River experiments, covered in Table III embraced 10 separate groups of the same seed which were divided into three main divisions as follows: Groups A to D exposed to freezing in the air, some soaked and others not; Groups E to H, soaked and unsoaked, frozen in the snow; and Group I, untreated check.

[4] PITTAUER, G. ÜBER DEN EINFLUSS VERSCHIEDENER BELICHTUNG UND EXTREMER TEMPERATUREN AUF DEN VERLAUF DER KEIMUNG FORSTLICHEN SAATGUTES. Centbl. Gesam. Forstw. 38: 157–172, 213–224, illus. 1912.

TABLE III.—*Results of freezing in air and snow, with and without soaking in water*

Group No.	No.	Treatment	Percentage of germination in 50 days
A	1	Freezing in air, not soaked	3.5
	2		4.0
	3	Tips cut	3.0
B	4	Freezing in air, all soaked before freezing	17.5
	5		6.5
	6	Tips cut	10.2
C	7	Freezing in air, soaked after freezing	6.5
	8		7.5
	9	Tips cut	11.5
D	10	Freezing in air, soaked before and after freezing	15.5
	11		11.0
E	12	Freezing in snow, not soaked	5.0
	13		5.5
	14	Tips cut	8.5
F	15	Freezing in snow, soaked before freezing	6.5
	16		6.0
	17	Tips cut	4.5
G	18	Freezing in snow, soaked after freezing	5.5
	19		5.5
	20	Tips cut	10.0
H	21	Freezing in snow, soaked before and after freezing	6.5
	22		7.5
I	23	Check tests, not frozen, not soaked	5.5
	24		6.5
	25	Tips cut	15.0

The soaked seed was immersed in cold water for 24 hours, and all of the samples except those used for checks were then exposed for 40 days, from February 22 to April 3, and tested in sand in the greenhouse. While complete greenhouse germination of this species requires 200 days, and is usually at a maximum after 100 days, these tests were concluded after 50 days in order to bring out more clearly the stimulating effect. A record of the daily temperatures during exposure of the seed is given below.

Temperature	Feb. 22-29	Mar. 1-15	Mar. 16-Apr. 3
Mean maximum	37	35	41
Minimum	21	25	28
High	41	44	48
Low	14	6	22

In Groups A to D, the highest germination (17.5 per cent) resulted from soaking 24 hours followed by air freezing; the next highest germination was 15.5 per cent, resulting from air freezing and soaking both before and after freezing; only 7.5 per cent germination resulted from soaking after air freezing; and germination in the check tests was from 5.5 per cent to 6.5 per cent. This indicates that germination may be hastened by this means and that soaking before freezing is more influential than soaking afterwards.

Better results were also obtained by soaking before freezing when snow was used, but germination was not so high as by air freezing. There was no noticeable difference between those not treated and those soaked after freezing.

Since the best results in this series of experiments exceeded those with the tips cut, which in previous tests had given the highest

germination, it is evident that a method is indicated by which large amounts of seed may be treated in bulk with very little labor and expense. After exposure the seed should promptly be mixed with moist bark-free sawdust and kept until the time of sowing, under temperatures between 35° and 40° F. Molding may be prevented effectively by stirring seed and sawdust every other day. If, after treatment, this seed should be allowed to hang or lie in storage until the time of sowing, it would most certainly deteriorate very rapidly, either by molding or by drying.

It should be noted that these results conform to those obtained by Pittauer, in that better germination was obtained by air than by snow freezing.

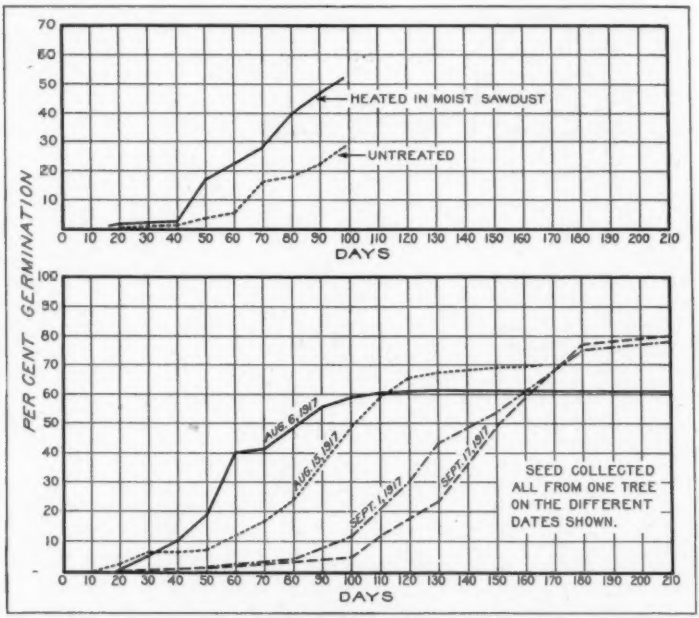

FIG. 2.—Greenhouse germination of western white pine seed, showing rate of germination during entire period of tests

PRELIMINARY TREATMENT IN MOIST SAWDUST

During the winter of 1916–17 a few samples of the western white pine seed were mixed with moist sawdust in a small wooden box and kept warm in the greenhouse. The seed was then covered with moist cotton and stirred thoroughly every day to prevent molding. This method, recommended by Johannes Rafn, has been in use for some time at the Danish seed control station in Copenhagen. The results of this treatment are given in Table IV and are shown graphically in Figure 2.

It is evident that germination may be nearly doubled by this means and that a three weeks' preliminary treatment is as good as or better than a longer or shorter time. This inexpensive method may

TABLE IV.—*Preliminary treatment in moist sawdust*

Manner and length of treatment		Germination in each period	
Water	Moist sawdust	50 days	100 days
Hours	*Days*	*Per cent*	*Per cent*
24	13	5.2	31.0
24	23	17.0	53.5
24	32	8.0	47.0
Untreated.	----------	2.8	29.5

then be recommended both for greenhouse sowing in sand and various germinators and for spring sowing in the nursery. It is necessary to maintain uniform medium moisture conditions by keeping the seed covered to avoid extremes of temperature, and by daily stirring to prevent development of mold spores. The soil temperature in these tests fluctuated between 60° and 90° F.

AFTER-RIPENING AND PERIOD OF REST

The investigations have revealed several methods by which white-pine germination may be hastened, but the question still remains unanswered whether the seed requires a period for after-ripening or for rest before active germination can take place. In order to throw light on this problem fresh seed was collected from a squirrel cache in a 250-year-old pine stand September 4, immediately extracted, and sowed soon afterwards, both in sand in the greenhouse and in the nursery. This seed had all the appearance of ripeness and was extracted at once without artificial heat.

Greenhouse sowing took place November 20. The sowing was of duplicate samples of 500 untreated seed and of seed with the tips cut as in the previous nursery tests. Of these the sample with the tips cut began to germinate December 7, or 17 days after sowing, and continued until complete with 74.6 per cent on April 20 following. The untreated sample began to germinate December 13 and was complete with 79.2 per cent July 10 following. Those untreated and sown in the nursery germinated very rapidly soon after warm weather developed, one lot reaching 79 per cent germination before June 19 and the other 83 per cent.

The prompt and complete germination of this seed in the greenhouse proved without doubt that after-ripening for this seed is of small moment. It did lead, however, to the belief that the seed was not entirely ripe. In order to prove this point further, seed was collected at successive intervals during the next good seed crop and tested.

The seed for this experiment was collected at intervals of about two weeks, from a well-developed tree about 75 years old, was extracted under ordinary September and October temperatures indoors without stove or other heat, and sowed in triplicate samples in sand in the greenhouse November 21. The results (given in Table V and fig. 2) are very interesting in showing a consistent and more rapid germination for the earlier lots, but in every case a higher final test for the seed of later collection.

TABLE V.—*Maturity test: Germination according to time of ripening*

Collection date	Greenhouse germination				Weight of 1,000 clean seeds
	50 days	100 days	150 days	210 days	
	Per cent	*Per cent*	*Per cent*	*Per cent*	*Grams*
Aug. 6	18.8	58.7	61.2	61.2	11.40
Aug. 15	7.0	48.7	60.2	70.7	13.35
Sept. 1	1.7	11.7	58.8	78.3	18.50
Sept. 17 *	1.3	5.0	49.0	80.0	17.35

* Seed falling after this date.

This more rapid germination rate of the earlier seed may have been caused by immature seed coat and the greater total germination of the later collection by a greater number of seed attaining ripeness.

When it is now considered that the sample in this maturity test collected September 1 gave a final germination of 78.3 per cent, which was as high as that in the after-ripening test collected September 4, there seems little ground for supposing that the seed was unripe or green except perhaps as regards the seed coat.

Sowing of fresh seed in the autumn will not always bring prompt spring germination, for if much rainy winter weather occurs, with light snow and temperatures of from 32° and 50° F., the seed will be injured.

Observations on the character of cones and seed showed that on on August 6 the cones were purple and the seed light brown, not filled out, and somewhat milky. On August 15 the cones were still purple and the seed light-colored but firmer and better filled. On September 1 there were also many light-colored seed, but all were filled out as well as those of September 17. The appearance of the seed and the weight on September 1 indicate that it was ripe at this date.

A microscopic examination was made of the structure and composition of the tegumentary system of the seed collected August 6 and September 17 from the same tree for the purpose of finding out whether the differences in time and amount of germination were due to greater development or hardening of the seed coat after August 6. In both samples the identical structure and composition were observed both by measurements and microchemical tests of the seed coat and peridermium thicknesses. It was apparent in cutting the sections, however, that the seed last collected had a harder coat. By soaking in concentrated sulphuric acid, the carpels of the seed collected August 6 decomposed sooner. The tests showed that the composition of the main hard seed-coat layer in both cases had not progressed beyond the formation of cellulose and that the cells of endosperm and embryo of both samples were filled with proteid. It was not necessary to use the microscope to determine that the endosperm of the early seed did not fill the carpel as well as that of the seed collected later.

Before concluding, it should be emphasized that a higher quality of seed has been produced from year to year by avoiding too high temperatures during extraction, and molding in storage. Investigations with this and the other species, as well as experiments con-

ducted with *Pinus sylvestris* at Eberswalde[5] and elsewhere, prove that a temperature above 115° F. is undesirable, particularly because of the greatly increased heating capacity of the moister air within the cones. The seed used in these experiments has been extracted promptly under temperatures below 90° F. and preserved from any other possible injury.

SUMMARY

This investigation has indicated that the delayed germination of western white-pine seed is not caused by any inherent physiological characteristic of the seed embryo or endosperm, such as after-ripening or the need for a period of rest, but by the impermeability of the seed coat, and that prompt germination may be obtained from unimpaired fresh seed by any of the following methods:

(1) Reduction of the seed coat by chemical corrosion, preferably by immersion for 45 minutes in concentrated sulphuric acid with thorough rinsing.

(2) Reduction of the seed coat by a mechanical abrasion or pearling process in such a manner that the seed is not cracked or subjected to much pressure.

(3) Soaking the seed in cold water for 24 hours and exposing it to air freezing in winter for at least 40 days.

(4) Burying the seed in moist bark-free sawdust, and maintaining it at a relatively warm temperature for three weeks, followed by immediate sowing.

In the absence of preliminary treatment, good germination may result from late fall sowing of good, newly collected seed, but rainy winter weather with temperatures above freezing may cause the seed to decay.

[5] HAACK, —. ÜBER DIE KEIMUNG UND BEWERTUNG DES KIEFERSAMENS NACH KEIMPROBEN. Ztschr. Forst u. Jagdw. 38: 441. 1906.

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